LIÈGE université
Gembloux
Agro-Bio Tech

Effects of cephalosporin treatment on the microbiota and resistance genes in milk and feces, and the presence of antibiotic heteroresistant strains

Author: Lei Dong
Promotors: Prof. Martine Schroyen & Prof. Jiaqi Wang

2023

COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Effects of cephalosporin treatment on the microbiota and resistance genes in milk and feces, and the presence of antibiotic heteroresistant strains

Lei Dong

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteurs: Prof. Martine Schroyen & Prof. Jiaqi Wang
Année civile: 2023

Abstract

Dairy cows with mastitis are frequently treated with antibiotics. However, the potential effects of β-lactam antibiotics, such as cephalosporins, on the milk and fecal microbiome is unknown. By studying both milk and feces, we aimed to assess the broader effects of cephalosporin use on the cow's overall microbial ecology. This approach allowed us to examine any potential transmission or changes in microbial populations from the gut (represented by feces) to the udder (represented by milk). Resistant bacteria are the main reason for failure of antibiotic treatment. However, even if bacteria have been identified as sensitive to antibiotics, these antibiotics are not always effective in treating bacterial infection diseases. The population can exhibit phenotypic heterogeneity, and heteroresistance is considered to be a crucial intermediate stage before the development of full resistance.

In the first experiment, the objective was to investigate the effect of a cocktail of two commonly used cephalosporins, ceftiofur and cefquinome, on the milk microbiota of 7 mastitis-affected dairy cows and the antibiotic resistance genes in the milk. Ceftiofur is a third-generation cephalosporin and cefquinome is a fourth-generation cephalosporin. Third-generation cephalosporins have a lower coverage against most gram-positive bacteria as compared to first- and second generation cephalosporins, but increased coverage against *Enterobacteriaceae*, *Neisseria spp*., and *Haemophilus influenzae*. Compared to third-generation cephalosporins, fourth-generation cephalosporins have an additional coverage against antibiotic-resistant gram-negative bacteria that produce beta-lactamases. In this first experiment, the milk samples were collected from 7 dairy cows at the period before medication (day 0), during medication (day 1, 2, and 3), during the withdrawal period (day 4, 6, and 8), and at several moments during the period after withdrawal (day 9, 11, 13, and 15). We applied 16S rRNA sequencing to explore the microbiota changes, and antibiotic resistance patterns were investigated by quantitative PCR. The microbiota richness and diversity in each sample were calculated using the Chao 1 (richness), Shannon (diversity), and Simpson (diversity) indices. The cephalosporins treatment lowered the Simpson diversity value at the period of withdrawal. Members of the *Enterobacter* genus were the most affected bacteria showing a significant reduction during the medication period which continued after. Meanwhile, antibiotic resistance genes in the milk were also influenced by antibiotic treatment. The cephalosporins treatment raised the proportion of *bla*TEM in milk samples at the period of withdrawal.

In the second experiment, the objective was to investigate the effects of ceftiofur and cefquinome on the fecal microbiota and antibiotic resistance genes in the feces of dairy cows with mastitis. The fecal samples were obtained from the same cows during the same period as in experiment 1. The fecal samples were collected from 8 dairy cows at the following periods: the start day (day 0), medication (day 1, 2, and 3), withdrawal (day 4, 6, 7, and 8), and recovery (day 9,

11, 13, and 15). Again, 16S rRNA gene sequencing was applied to explore the changes in microbiota, and qPCR was used to investigate the antibiotic resistance genes. The cephalosporin treatment significantly decreased the microbial diversity and richness, indicated by the decreased Shannon and Chao 1 indexes, respectively ($p < 0.05$). The relative abundance of *Bacteroides*, *Bacteroidaceae*, *Bacteroidales*, and *Bacteroidia* increased, and the relative abundance of *Clostridia*, *Clostridiales*, *Ethanoligenens*, and *Clostridium* IV decreased at the withdrawal period. However, we observed that the relative abundance of the bovine mastitis-related bacterial genera, such as *Blautia*, *Curvibacter*, *Bradyrhizobium*, etc, significantly decreased at the medication period in milk, but did not change in the feces. The cephalosporin treatment increased the relative abundance of β-lactamase resistance genes (*bla*TEM and *cfx*A) at the withdrawal period ($p < 0.05$) in feces, thus the relative abundance of *bla*TEM increased in both milk and fecal samples during this withdrawal period.

In the first experiment, the cephalosporin treatment did not have a significant effect on the relative abundance of *Staphylococcus* in milk. However, when analyzing the *Staphylococcus* isolated from the milk samples, they were almost all completely sensitive to ceftiofur (68/70, 97%). If the *Staphylococcus* isolated from milk samples are truly sensitive to ceftiofur, the cephalosporin treatment should theoretically significantly reduce the relative abundance of *Staphylococcus*. Therefore, we hypothesized that *Staphylococcus* may have developed antibiotic heteroresistance (HR). So, in the third experiment, we studied the prevalence of ceftiofur heteroresistant *Staphylococcus* isolates. In total, 151 *Staphylococcus* isolates were collected from milk from mastitis suffering cows from the major dairy-production areas of China, including 70 different *Staphylococcus* strains isolated from milk samples from the first experiment. To avoid duplicates, *Staphylococcus* isolates were considered unique if strains from the same milk sample differed at the species level by 16S rRNA sequencing. Among the isolates, 15 strains (15/151, 9.9%) showed heteroresistance by the disk diffusion method, and, of those 15, three strains (3/15, 20%) exhibited heteroresistant phenotypes by the PAP method. Two of these heteroresistant strains were unstable, as the minimal inhibitory concentrations (MICs) decreased after 1-week daily culture. Whole-genome sequencing displayed that, for strains with heteroresistant phenotypes, there were single nucleotide polymorphisms in the *mec*A gene, leading to different protein sequences, which might be associated with ceftiofur heteroresistance. There were two extra mutations in the heteroresistant stable isolate (D12-4), which might have resulted in the formation of a stable resistant subpopulation in heteroresistant *Staphylococcus*.

In summary, the treatment of cephalosporins, of which ceftiofur is one well-known example, led to a change in the milk and fecal microbiota and increased β-lactamase resistance genes in both milk and feces at the time of withdrawal period. These findings also raise concerns about the emergence of ceftiofur-heteroresistant *Staphylococcus* isolates and the application of ceftiofur as therapy

for the treatment for *Staphylococcus*-induced mastitis in dairy cows.

Keywords: dairy cow mastitis, cephalosporin, milk, feces, *Staphylococcus*, heteroresistance.

Résumé

Les vaches laitières atteintes de mammite sont fréquemment traitées avec des antibiotiques. Cependant, les effets potentiels des antibiotiques β-lactamines, tels que les céphalosporines, sur le microbiome du lait et des matières fécales sont inconnus. En étudiant à la fois le lait et les fèces, nous avons cherché à évaluer les effets plus larges de l'utilisation des céphalosporines sur l'écologie microbienne globale de la vache. Cette approche nous a permis d'examiner toute transmission ou modification potentielle des populations microbiennes de l'intestin (représenté par les fèces) à la mamelle (représentée par le lait). Les bactéries pathogènes résistantes sont la principale cause d'échec du traitement antibiotique. Cependant, même si des bactéries ont été identifiées comme sensibles aux antibiotiques, ces derniers ne sont pas toujours efficaces pour traiter les maladies infectieuses bactériennes. La population peut présenter une hétérogénéité phénotypique et l'hétérorésistance est considérée comme une étape intermédiaire cruciale avant le développement d'une résistance totale.

Dans la première expérience, l'objectif était d'étudier l'effet d'un cocktail de deux céphalosporines couramment utilisées, le ceftiofur et le cefquinome, sur le microbiote du lait de 7 vaches laitières atteintes de mammite et les gènes de résistance aux antibiotiques dans le lait. Le ceftiofur est une céphalosporine de troisième génération et le cefquinome est une céphalosporine de quatrième génération. Les céphalosporines de troisième génération ont une couverture inférieure contre la plupart des bactéries gram-positives par rapport aux céphalosporines de première et deuxième génération, mais une couverture accrue contre les *Enterobacteriaceae*, *Neisseria* spp. et *Haemophilus influenzae*. Par rapport aux céphalosporines de troisième génération, les céphalosporines de quatrième génération ont une couverture supplémentaire contre les bactéries gram-négatives résistantes aux antibiotiques qui produisent des bêta-lactamases. Dans cette première expérience, les échantillons de lait ont été prélevés sur 7 vaches laitières à la période précédant la médication (jour 0), pendant la médication (jours 1, 2 et 3), pendant la période d'attente (jours 4, 6 et 8), et à plusieurs moments de la période suivant l'attente(jours 9, 11, 13 et 15). Nous avons appliqué le séquençage de l'ARNr 16S pour explorer les changements du microbiote, et les modèles de résistance aux antibiotiques ont été étudiés par PCR quantitative. La richesse et la diversité du microbiote dans chaque échantillon ont été calculées à l'aide des indices Chao 1 (richesse), Shannon (diversité) et Simpson (diversité). Le traitement aux céphalosporines a abaissé la valeur de la diversité de Simpson à la période de sevrage. Les membres du genre Enterobacter étaient les bactéries les plus touchées, montrant une réduction significative pendant la période de traitement qui s'est poursuivie par la suite. Pendant ce temps, les gènes de résistance aux antibiotiques dans le lait ont également été influencés par le traitement antibiotique. Le traitement aux céphalosporines a augmenté la proportion de *bla*TEM dans les échantillons de lait au moment du sevrage.

Dans la deuxième expérience, l'objectif était d'étudier les effets du ceftiofur et

de la cefquinome sur le microbiote fécal et les gènes de résistance aux antibiotiques dans les fèces de vaches laitières atteintes de mammite. Les échantillons fécaux ont été prélevés sur les mêmes vaches au cours de la même période que dans l'expérience 1. Les échantillons fécaux ont été prélevés sur 8 vaches laitières aux périodes suivantes : le jour de début (jour 0), la médication (jours 1, 2 et 3), l'attente (jours 4, 6, 7 et 8) et la récupération (jour 9, 11, 13 et 15). Encore une fois, le séquençage du gène de l'ARNr 16S a été appliqué pour explorer les changements dans le microbiote, et la qPCR a été utilisée pour étudier les gènes de résistance aux antibiotiques. Le traitement aux céphalosporines a significativement diminué la diversité et la richesse microbiennes, indiquées par la diminution des indices de Shannon et de Chao 1, respectivement ($p < 0,05$). L'abondance relative des *Bacteroides*, *Bacteroidaceae*, *Bacteroidales* et *Bacteroidia* a augmenté, et l'abondance relative des *Clostridia*, *Clostridiales*, *Ethanoligenens* et *Clostridium IV* a diminué au moment de la période de retrait. Cependant, nous avons observé que l'abondance relative des genres bactériens liés à la mammite bovine, tels que *Blautia*, *Curvibacter*, *Bradyrhizobium*, etc., diminuait significativement à la période de médication dans le lait, mais ne changeait pas dans les fèces. Le traitement aux céphalosporines a augmenté l'abondance relative des gènes de résistance aux β-lactamases (*bla*TEM et cfxA) à la période d'attente ($p < 0,05$) dans les matières fécales, ainsi l'abondance relative de *bla*TEM a augmenté dans les échantillons de lait et de matières fécales pendant cette période d'attente.

Dans la première expérience, le traitement aux céphalosporines n'a pas eu d'effet significatif sur l'abondance relative de *Staphylococcus* dans le lait. Cependant, lors de l'analyse des *Staphylococcus* isolés des échantillons de lait, ils étaient presque tous complètement sensibles au ceftiofur (68/70, 97 %). Si les *Staphylococcus* isolés dans les échantillons de lait sont réellement sensibles au ceftiofur, le traitement par céphalosporine devrait théoriquement réduire de manière significative l'abondance relative des *Staphylococcus*. Par conséquent, nous avons émis l'hypothèse que *Staphylococcus* pourrait avoir développé une hétérorésistance aux antibiotiques (HR). Ainsi, dans la troisième expérience, nous avons étudié la prévalence des isolats de *Staphylococcus* hétérorésistants au ceftiofur. Au total, 151 isolats de Staphylococcus ont été collectés à partir de lait de vaches souffrant de mammite dans les principales zones de production laitière de Chine, dont 70 différentes souches de Staphylococcus isolées à partir d'échantillons de lait de la première expérience. Pour éviter les doublons, les isolats de Staphylococcus ont été considérés comme uniques si les souches provenant du même échantillon de lait différaient au niveau de l'espèce par le séquençage de l'ARNr 16S.Parmi les isolats, 15 souches (15/151, 9,9 %) ont montré une hétérorésistance par la méthode de diffusion sur disque et, parmi ces 15, trois souches (3/15, 20 %) ont présenté des phénotypes hétérorésistants par la méthode PAP. Deux de ces souches hétérorésistantes étaient instables, car les concentrations minimales inhibitrices (CMI) diminuaient après 1 semaine de culture quotidienne. Le séquençage du génome entier a montré que, pour les

souches présentant des phénotypes hétérorésistants, il existait des polymorphismes nucléotidiques uniques dans le gène mecA, conduisant à différentes séquences protéiques, qui pourraient être associées à l'hétérorésistance au ceftiofur. Il y avait deux mutations supplémentaires dans l'isolat stable hétérorésistant (D12-4), qui auraient pu entraîner la formation d'une sous-population résistante stable chez *Staphylococcus* hétérorésistant.

En résumé, le traitement aux céphalosporines, dont le ceftiofur est un exemple bien connu, a entraîné une modification du microbiote laitier et fécal et une augmentation des gènes de résistance aux β-lactamases dans le lait et les fèces au moment du temps d'attente. Ces résultats soulèvent également des inquiétudes quant à l'émergence d'isolats de *Staphylococcus* hétérorésistants au ceftiofur et à l'application du ceftiofur comme thérapie pour le traitement de la mammite induite par *I* chez les vaches laitières.

Mots-clés : mammite de vache laitière, céphalosporine, lait, matières fécales, *Staphylococcus*, hétérorésistance.

Acknowledgments

First of all, I would like to express my heartfelt thanks to my thesis jury members who have ever helped me with this paper: Prof. Martine Schroyen, Prof. Jiaqi Wang, Prof. Nadia Everaert, Prof. Jérôme Bindelle, Prof. Jean-Luc Hornick, and Prof. Damien Thiry for their insightful comments and encouragement.

My sincere thanks and appreciation go to my supervisors, Prof. Martine Schroyen and Prof. Jiaqi Wang. Thank you for guiding me both professionally and personally, for continuously supporting my PhD study and related research, and for their trust in my ability to conduct my research properly in a new field.

I would also like to thank Pro. Nan Zheng, Dr. Lu Meng, and Dr. Huimin Liu for the help of experimental design and thesis writing. I learned many important aspects of conducting a research project successfully to its end, and thank them for the long and instructive discussions that we had over the years about and around my PhD project.

A large part of my work would not been possible to finish without efficient collaboration with Yangdong Zhang, Shengguo Zhao, Yanan Gao, Mengru Xing, Xiaoyu Bao, Haiyan Hu, Chenqing Wu, Sijia Liu, Huiyue Zhong, and Zhanbo Xiong during my stay at the Institute of Animal Sciences of the Chinese Academy of Agricultural Sciences (CAAS), and Cécile Russo, Sylvie Mabille, Chong Li, Siegien Pawel during my stay at the lab of Precision Livestock and Nutrition Unit of Gembloux Agro-Bio tech. I will be forever appreciative for the wonderful experiences I have had throughout my time in Beijing and Gembloux.

Sincere gratitude is given for the China Scholarship Council (CSC)'s financial support of this thesis as well as the support provided by CAAS and the University of Liège (ULg) for the program.

In addition, many thanks go to my parents, my sister, and my boyfriend, for their unfailing love and unwavering support. My deepest gratitude is expressed to my family for their encouragement and support in getting this degree.

Finally, I want to thank everyone who took the time to read this thesis and gave me valuable advice that will help me in my future studies.

Lei Dong
December, 2023 in Gembloux, Belgium

Contents

List of figures

List of tables

List of abbreviations

ABC: ATP-binding cassette
ACP: amoxicillin and clavulanate potassium
AMK: amikacin
AMP: ampicillin
AMR: antimicrobial resistance
ARB: antibiotic resistant bacteria
ARGs: Antibiotic resistance genes
AST: antimicrobial susceptibility testing
BMD: broth microdilution method
CCFA: ceftiofur crystalline free acid
CEF: ceftiofur
CEP: cephalothin
CFU: colony-forming unit
CHC: ceftiofur hydrochloride
CHS: CEF-heteroresistant Staphylococcus
CIP: ciprofloxacin
CLI: clindamycin
CLSI: Clinical Laboratory Standards Institute
CMT: California mastitis test
CONT: control group
CRO: ceftriaxone
DHPS: dihydropteroate synthase
Dntp: deoxyribonucleoside triphosphate
DOX: doxycycline
EARS-Net: European Antimicrobial Resistance Surveillance Network
EARSS: European Antimicrobial Resistance Surveillance System
ERY: erythromycin
ESC: extended-spectrum cephalosporin
ESCR-E: extended-spectrum cephalosporin resistant Enterobacterales
ESBLs: Extended-spectrum β-lactamases
ESVAC: European Surveillance of Veterinary Antimicrobial Consumption
EU: European Union
EUCAST: European Committee on Antimicrobial Susceptibility Testing
FAO: Food and Agriculture Organization
FDA: Food and Drug Administration
FLO: florfenicol
GEN: gentamicin
GNB: Gram-negative bacteria
HGT: horizontal gene transfer

HR: heteroresistance
hVISA: heteroresistance vancomycin intermediate S. aureus
KOD: kodakaraensis
LEfSe: Linear discriminant analysis effect
LOQ: limit of quantification
MATE: Multidrug and Toxic Compound Extrusion
MDR: multidrug-resistant
MFS: Master Facilitator Superfamily
MGE: mobile genetic elements
MHA: Mueller-Hinton agar
MIAs: medically important antimicrobials
MIC: minimum inhibition concentration
MRSA: Methicillin-resistant Staphylococcus aureus
OMP: outer membrane porin
OTC: oxytetracycline
OUT: operational taxonomic units
OXA: oxacillin
PABA: p-aminobenzoic acid
PAP: Population analysis profiling
PBPs: penicillin-binding proteins
PCR: polymerase chain reaction
PCST: pediatric compound sulfamethoxazole tablets
PDL: Prescription Drug List
PEN: penicillin
PMF: proton motive force
PPG: procaine penicillin G
RIF: rifaximin
RND: Resistance Nodule and Cell Division
SHV: sulfhydryl reagent variable
SMR: small multidrug resistance
SUL: sulfisoxazole
THFA: tetrahydrofolic acid
TOB: tobramycin
TUL: tulathromycin
TZP: piperacillin/tazobactam
VAN: vancomycin
VGT: vertical gene transfer
WHO: World Organization for Health
WOAH: World Organization for Animal Health

Chapter 1

General introduction

Chapter I. General introduction

1.1 The emergence of antibiotic resistance

Before the introduction of penicillin some anti-infectives such as arsenic and sulfonamides, drugs made by chemically tinkering with synthetic dyes, and many disinfectants made with metal ions, such as mercury or copper, that are toxic to bacteria, were in use to prevent or treat infections. In 1928, Alexander Fleming recognized the significance of the antibacterial properties of Penicillium mold, but he never went on to purify or test the substance, which he called Penicillin, any further. It was a decade later that Fleming's findings caught more interest and the first tests on mice were done by Florey and Chain (Ligon et al., 2004). Thereafter, the use of this and other antibiotics escalated in human and animal medicine. This increase in antibiotic use was due to two major changes: wartime troop movement in the 1940s and the intensification of the poultry industry (Landecker et al., 2019). Today, antibiotics are generally classified as broad-spectrum or narrow-spectrum antibiotics according to their spectrum of antibacterial activity. Broad-spectrum antibiotics (i.e., doxycycline, azithromycin, amoxicillin and clavulanic acid, mupirocin, and fluoroquinolones) target a variety of gram-positive and gram-negative bacteria, while narrow-spectrum antibiotics (i.e., vancomycin, fidaxomicin, and sarecycline) target only a limited number of clinically relevant bacteria. Furthermore, antibiotics are divided into bactericidal (i.e., they kill bacteria) and bacteriostatic (i.e., they only inhibit the growth or proliferation of bacteria) categories based on their mechanism of action. For example, tetracyclines are generally bacteriostatic, while fluoroquinolones are bactericidal (Grada et al., 2021).

The use of antibiotics in agriculture varies across regions and countries, but some antibiotics that have been banned in certain countries, mostly developed countries, are still currently being used in most developing countries. It is reported that the total antibiotic consumption in livestock in 2010 was 63151 tons (Van Boeckel et al., 2015). They estimated that the antibiotic consumption would rise by 67% by 2030, and roughly double in the BRICS countries that represent Brazil, Russia, India, China and South Africa. This rise is driven by the growing consumer demand for animal products and the need for maintaining the necessary animal health standards and high productivity levels on large scale farms. Antibiotic resistance is an inherent side effect related to the overuse, abuse, or continuous use of antibiotics (Williams-Nguyen et al., 2016). The antibiotic crisis is ascribed to the abuse of antibiotics, which are finally discharged into the environment and remain in livestock products.

The lack of effective supervision and control is the main reason of antibiotic resistance (Tang et al., 2016). The Ministry of Health in China issued strategies to limit the use of antibiotics in humans and animals, but the government failed to control the sales of antibiotics, making them available everywhere in the market.

The irrational use of antibiotics in animals, humans, and environments, results in antibiotic residues or the persistence of antibiotic resistance genes (ARGs) in different environments. These environments, including soil, water, hospital, and farm waste, have been regarded as vital reservoirs and sources of antibiotic resistance dissemination (Figure 1-1) (Xiao et al., 2016). Antibiotic resistance has its origins in mutations in genes encoded on microbial chromosomes, and since genetic material can be exchanged between organisms, so can these mutations. This process therefore can provide host cells and their progeny with new genetic material encoding antibiotic resistance. Antibiotics exert selective pressure on the emergence of antibiotic resistance and induce the transfer of resistance genes among microorganisms (Holmes et al., 2016).

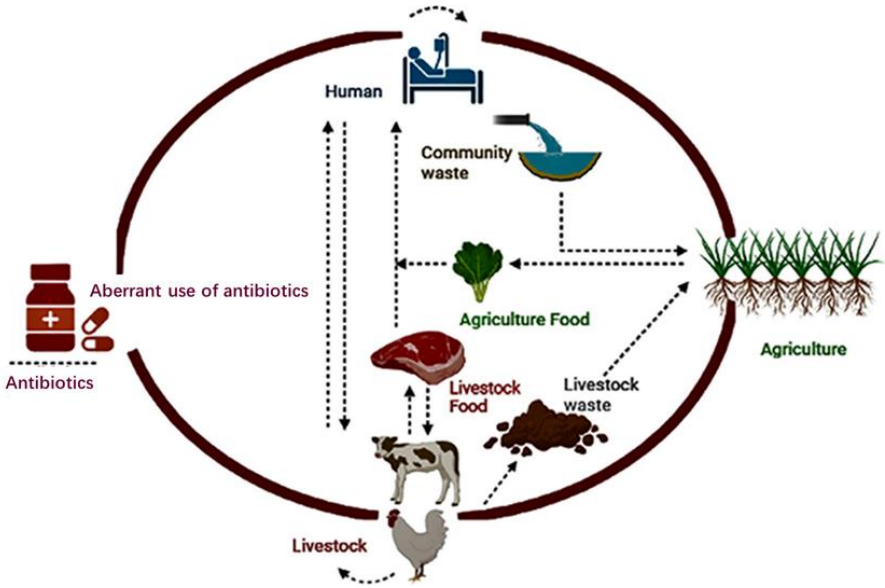


Figure 1-1. The antibiotic resistance dissemination in different environment

1.1.1 Intrinsic antibiotic resistance

Bacteria are generally considered to be inherently resistant according to clinical definition if their infection cannot be treated with a given antibiotic (Olivares et al., 2013). In the ecological definition of resistance, the "intrinsic resistance" has been defined as a group of elements that directly or indirectly contribute to antibiotic resistance, the presence of which is independent of previous antibiotic exposure and is not due to horizontal gene transfer (HGT) (Paquola et al., 2018). Antibiotic resistance can be mediated through pre-existing phenotypes in natural bacterial populations. During evolution, bacterial cells accumulate genetic errors in existing genes (on the chromosomes or on plasmids). Gene duplication error

rates are fairly low, so typically one in a thousand growing bacteria will introduce errors (mutations) into the genome. About one in a billion bacteria are mutants that can grow faster or tolerate higher concentrations of antibiotics than their predecessors. When these bacterial mutants are exposed to antibiotics, those with antibiotic resistance genes will have an increased prevalence (Dantas et al., 2014). Thereafter the resistance genes can be transferred to descendant cells through vertical gene transfer (VGT), resulting in innate, intrinsic or natural resistance (Figure 1-2a).

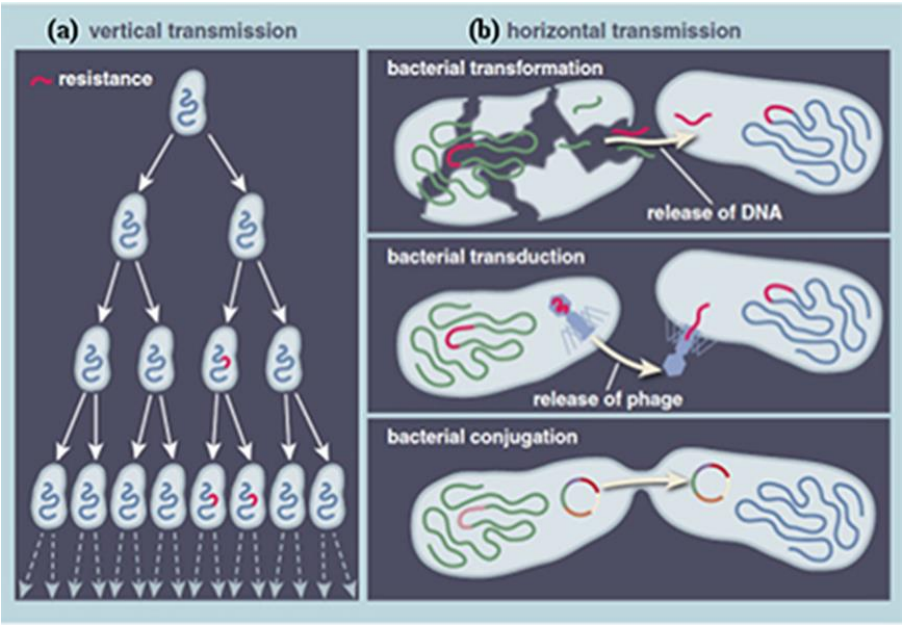


Figure 1-2. Principal modes of transmission involved in the evolution and development of antibiotic resistance. (a) Vertical transmission (VGT) of intrinsic antibiotic resistance (b) Horizontal transmission (HGT) leading to acquired antibiotic resistance (Dantas et al., 2014).

1.1.2 Acquired antibiotic resistance

Acquired resistance involves genetic exchange within and between bacterial species (Holmes et al., 2016). It implies HGT and the acquisition of new ARGs carried on mobile genetic elements (MGE), such as plasmids, integrons, transposons, insertion sequences, and phage-associated elements (Figure 1-2b). These genetic materials are passed on via conjugation (transfer of DNA from donor to recipient bacteria by cell-to-cell contact, with the help of fertility factors called pili), transformation (naked DNA present in the environment is taken up by the recipient cells) or transduction (bacteriophages act as vectors and insert DNA into recipient cells) (Dantas et al., 2014). HGT breaks the boundaries of kinship

and enables gene exchange between different species (Paquola et al., 2018). It is reported that bacterial antibiotic-resistant HGT plays an important role in the evolution and spread of multidrug resistance (Lerminiaux et al., 2019).

Bacteria have developed mechanisms to evade antibiotics by adopting transient expression of resistance genes. This strategy involves temporarily increasing resistance in a subset of cells, which undermines the effectiveness of antibiotics and leads to chronic and difficult-to-treat infections (Mulcahy et al., 2010). It is important to recognize that transient resistance is not caused by genetic changes, rather cells use phenotypic variability or induce gene expression to generate a resistant phenotype (Levin et al., 2006).

1.1.3 Detection methods of antibiotic resistance

Bacteria can be sensitive to a given antibiotic, and thus be treated with that antibiotic, they can be resistant and thus not be treated, or they can be intermediate resistant, which means that they can be treated, but only with a certain minimum dosage of a given antibiotic. Antimicrobial susceptibility testing methods such as the MIC (minimum inhibitory concentration) determination (Vijayakumar et al., 2011) (Figure 1-3), the disc diffusion method (Parvin et al., 2020) (Figure 1-4) and the Etest (Bailey et al., 2018) (Figure 1-5) as procedures for defining isolates as sensitive, resistant, or intermediate resistant to any antibiotic are generally accepted worldwide. MIC is the lowest concentration of an antibiotic expressed in mg/L (μg/mL), that, under strictly controlled in vitro conditions, completely prevents visible growth of the test isolate of the bacteria (EUCAST, 2000). With the MIC determination method, a dilution series of antibiotics is tested in the presence of the potential target bacterial isolates to determine the MIC (Figure 1-3). With the disc diffusion method, the surface of an agar plate is inoculated with bacteria, and a paper disk containing the antibiotic is applied to the agar and the plate is incubated (Figure 1-4). If the antibiotic stops the bacteria from growing or kills the bacteria, there will be an area around the disk where the bacteria have not grown enough to be visible. This is called the zone of inhibition. The sensitivity of bacterial isolates to each antibiotic can then be judged by comparing the size of these zones of inhibition (EUCAST, 2021). The Etest, a test developed by bioMérieux, France, is an antibiotic susceptibility test that quantitatively measures the MIC values of an antibiotic (Singh et al., 2012) (Figure 1-5). The Etest uses plastic strips containing a continuous gradient of a specific antibiotic on one side and a code for the associated concentration on the other side. The principle is similar to the disc diffusion method.

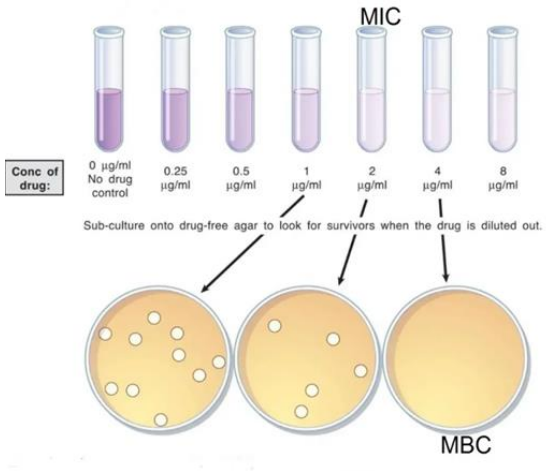


Figure 1-3. Example of a dilution plate to determine the MIC (µg/mL) value of an antibiotic against bacterial isolates. MBC means minimum bactericidal concentration (Vijayakumar et al., 2011)

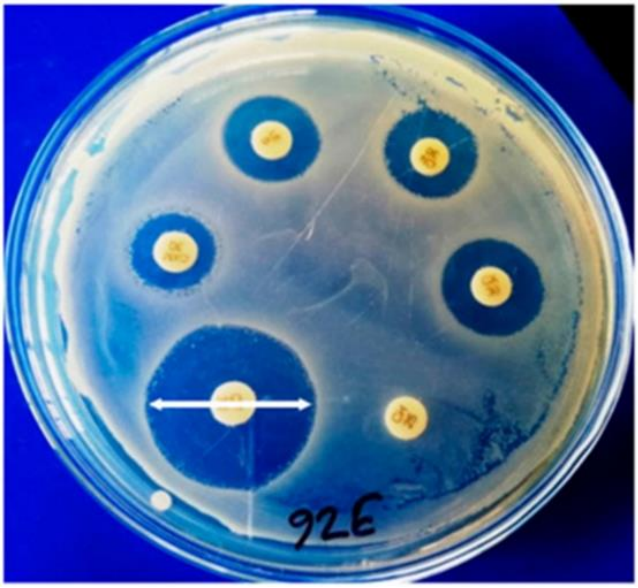


Figure 1-4. Example of an antimicrobial susceptibility test of bacteria by the disc diffusion method, showing a zone of inhibition (↔) (Parvin et al., 2020).

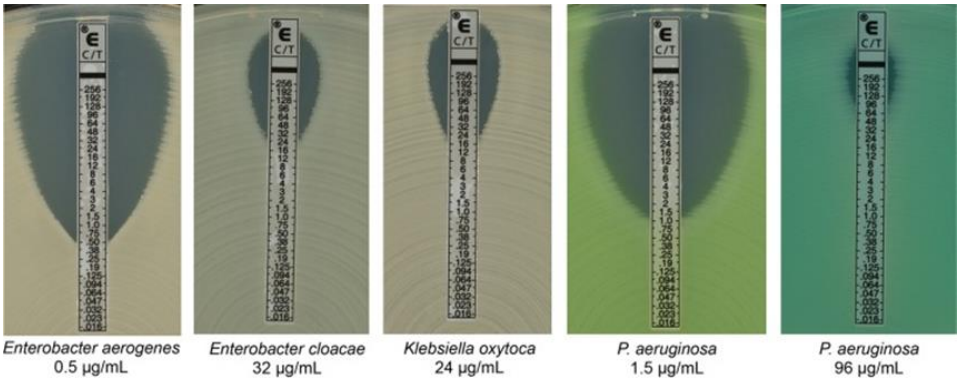


Figure 1-5. Examples of Etests against different bacterial isolates. Etest strips were placed onto agar plates. Plates are read after an incubation of 16 to20h in ambient air at 35 ± 2°C. The MIC results are read as where the zone of inhibition ellipse intersects the Etest strip; if the ellipse intersects between two MIC values, the higher of the two values is reported (Bailey et al., 2018).

1.2 The harm of antibiotic resistance

1.2.1 The harm to the ecological environment

Antibiotics are used to protect human health and inhibit the emergence of animal disease. However, antibiotics freely available in the environment are a big concern. These antibiotics are found in the environment because of their low absorption capacity in the intestines (Akram et al., 2017; Callaway et al., 2021). The undigested antibiotics are discharged through urine and feces into the environment (Sarmah et al., 2006). Antibiotics can also be released into the environment through agricultural activities such as the use of animal manure as fertilizer or the disposal of wastewater (Martinez et al., 2017; Quaik et al., 2020). These practices have resulted in the contamination of different habitats with large concentrations of antibiotics. Since antibiotics are inhibitors of bacterial growth, this situation has implications for the structure and the activity of bacterial populations (Martinez et al., 2017). Antibiotic residues can reduce or eliminate microbial communities or expand antibiotic resistant bacteria (ARB) (Grenni et al., 2018). The degradation of the soil microbiome can affect microbial processes such as the mineralization and decomposition of organic matter (Kumar et al., 2005). According to the report, antibiotic pollution in aquatic environment has the potential to decrease overall microbial diversity, including the taxa responsible for carbon cycling and primary productivity (Kraemer et al., 2019). Additionally, antibiotics can impact bacterial enzyme activities, such as dehydrogenase, phosphatase, and urease, which serve as critical indicators of soil activity (Cycoń et al., 2019). Furthermore, the disturbance caused by antibiotics on microbial communities can result in an elevated abundance of parasites and bacteria in both soil and water environments. It is reported that the presence of antibiotics in

aquatic environments has led to an increased frequency of toxic cyanobacterial species, leading to the eutrophication in freshwater environments (Drury et al., 2013).

1.2.2 The harm to husbandry

Since the 1960s, antibiotics have been widely used as growth promoters for food-producing animals (Hassan et al., 2018). The problem with uncontrolled doses of growth-promoting antibiotics is that they create conditions that favor the selection of ARB, which then pollute the environment (Meek et al., 2015). The presence of ARB may enhance the transfer of ARGs to the gut bacteria in host animals. Moreover, the presence of antibiotics can stimulate HGT in some pathogens; for example, resistance to azithromycin, ciprofloxacin, or tigecycline has been observed in *Enterococcus faecalis* and *P. aeruginosa* (Zalewska et al., 2021).

1.2.2.1 The harm to aquaculture husbandry

Aquaculture production accounts for almost half of the global consumption of fish and fish-by products, posing a shift to intensified and semi-intensive production practices. As a result, the use of antibiotics for both therapeutic and non-therapeutic purposes have increased dramatically (Palma et al., 2020). This inevitably led to the customary use of antibiotics, leading to a strong selection pressure favoring the emergence and selection of antimicrobial resistance (AMR) strains, and the subsequent transmission of AMR through various routes such as food, feed, and environment (Ryu et al., 2012; Cabello et al., 2013; Santos et al., 2018). To date, there are no antibiotics specifically designed for aquaculture. Inappropriate use of antibiotics has been linked to a reduced ability of fish to efficiently metabolize the administered drugs. Therefore, antibiotic residues persist in fish meat for a prolonged time, facilitating the spread to terrestrial ecosystems through the food chain. Furthermore, it is estimated that 70-80% of the active compounds are eliminated by feces, enabling the diffusion of antibiotics through wastewater; thus, affecting multiple ecosystems (Cabello et al., 2016). ARGs have been reported to be transferred between aquatic bacteria that are pathogenic to both fish and humans (Ryu et al., 2012). For the farmed shellfish, deadly pathogens such as *Vibrio* and *Salmonella* may acquire resistance through horizontal transfer. For example, the fish pathogens *Vibrio* and *Lactococcus* can transfer tetracycline ARGs to human *Escherichia coli* and *Enterococcus faecalis* (Neela et al., 2009). The World Organization for Health (WHO), the World Organization for Animal Health (WOAH, formerly the Office International des Epizooties), and the Food and Agriculture Organization (FAO) expert consultation on *Antimicrobial Use and Antimicrobial Resistance in Aquaculture* (2006) concluded that public health hazards associated with the use of antibiotics in aquaculture include the development and spread of ARB and ARGs, and the emergence of antibiotic residues in aquaculture products (FAO/OIE/WHO, 2006).

1.2.2.2 The harm to livestock animal husbandry

As earlier stated, in the early years of antibiotic use in livestock, antibiotics were used not only for disease treatment, but also often merely as growth promoters. The use of antibiotics in veterinary medicine is however ideally

associated with the treatment of infectious diseases. Therapeutic interventions should be designed following accurate pathogen identification and antimicrobial susceptibility testing (AST) (Aarestrup et al., 2005). Nevertheless, it is a common method to extend the antimicrobial treatment to the entire livestock herd to limit the pathogen spread, leading to an overuse of antibiotics as non-infected animals are also administrated with the antibiotics (Economou et al., 2015). A validated growth-based AST, based on the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or the Clinical Laboratory Standards Institute (CLSI), is currently recommended to guide the antimicrobial therapy (Vasala et al., 2020). Most recurrent infections on cattle farms are related to shipping fever, bovine pneumonia, and diarrhea, requiring heavy use of common antibiotics, such as penicillin, quinolone, gentamicin, and tylosin (McEwen et al., 2002). In addition, broad-spectrum antibiotics are often used to treat liver infections, while narrow-spectrum antibiotics, such as beta-lactams are the first choice for streptococcal mastitis (Cameron et al., 2016). In the food-producing animal industry, antibiotics have been seen to affect the functions of gut bacteria and can temporarily increase antibiotic resistance in the microbiome (Broom et al., 2017). The feces from numbers of animals raised under intensive conditions is often spread on pasturelands as fertilizer. Groundwater, streams and other waterways contaminated by these wastes may also facilitate the spread of bacteria that carry antibiotic resistance traits (McEwen et al., 2002). Some countries have officially banned the use of antibiotics in livestock except for medical use, others not yet. A study on antibiotic use in South East Asia in 2016 showed that, in addition to feed, farmers in this region applied 46 mg of different antibiotics per kilogram (kg) of live pigs and 52-276 mg per kg of live chickens per year (Nhung et al., 2016). It is estimated that approximately 84% of antibiotics administered in chicken farms located in the Mekong Delta region were solely used for preventive intentions (Nhung et al., 2016). The non-medical treatment of antibiotics, such as treating entire extensive animal production facilities in response to the illness of a single animal, has contributed to the proliferation of ARBs within the animal production chain (Zalewska et al., 2021).

1.2.3 The harm to food safety and human health

The WHO, WOAH and FAO have shown that the non-human use of all kinds of antibiotics may lead to harmful results for human health (So et al., 2015). Livestock animals, fish and vegetables are considered large hosts of ARB, and the food production chain is an ecosystem consisting of different ecological niches in which a large amount of antibiotics is used and many bacteria co-exist (Acar et al., 2006). ARB can be spread along the food chain through direct or indirect contact. The rapid spread of ARB between hosts is enhanced by the immediate exposure of humans to animals and biological materials such as blood, urine, feces, milk, saliva, and semen. Occupationally exposed workers, such as veterinarians, farmers, abattoir workers, and food handlers, and those in direct contact with them, are at high risk of becoming colonized or infected with ARB (Marshall et al., 2011). Furthermore, there is a potential for indirect exposure of the human population to ARB and ARGs through contact with or consumption of

contaminated food items, including meat, eggs, milk, and dairy products. The transmission of ARB and ARGs via the food chain represents a widespread and intricate mode of dissemination (Figure 1-6). Recent studies have highlighted the presence of significant quantities of ARB and ARGs in various food products derived from different animals such as cattle, poultry, swine, sheep, and goats (Coetzee et al., 2016; Yang et al., 2022). These food products include ready-to-eat meat, cooked meat, and raw milk. These ARB and ARGs can interfere with the action of prescribed medications, cause allergic reactions, interfere with the natural gut flora, or lead to the further evolution of ARB that can eventually lead to health problems such as toxic effects, hepatotoxicity, nephropathy, mutagenicity, carcinogenicity and antibiotic resistance (Chen et al., 2019).

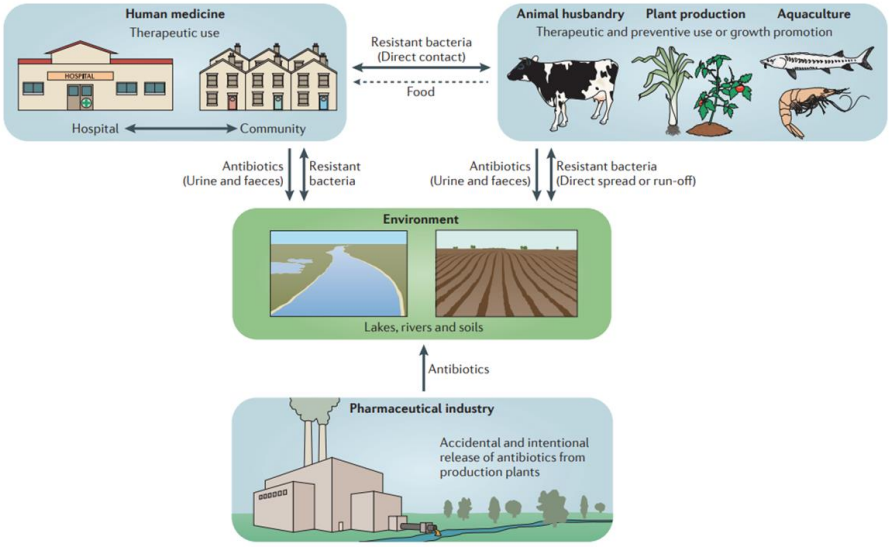


Figure 1-6. Antibiotic use and different transmission routes of antibiotic resistance in the food chain (Andersson et al., 2014).

In the following sections we will give examples of the different mechanisms on how antibiotic resistance can occur, what the current state is with regard to milk from dairy cattle and how to detect it. It is paramount to understand that there are various antibiotic resistance mechanisms and the potential spread of antibiotic resistance can be quite complex.

1.3 Mechanisms of antibiotic resistance

1.3.1 Target modifications or genetic mutations

Antibiotics can inhibit bacterial proliferation through cell destruction or growth inhibition. A genetic mutation or modification of the target site will interfere with the normal bond between antibiotic and pathogen, thereby affecting the effect of the antibiotic. During infection, there is often a large and diverse population of pathogens, and if a single point mutation in a gene encoding an antibiotic target

can confer antibiotic resistance, strains with this mutation can subsequently proliferate (Blair et al., 2015). For example, β-Lactams are an important class of antibiotics whose members contain a β-lactam ring and inhibit peptidoglycan synthesis by covalently binding to the active site serine of bacterial penicillin-binding proteins (PBPs). PBPs are located on the bacterial cytoplasmic membrane and play a role in the synthesis of cell wall peptidoglycan. PBPs can promote the synthesis and maintenance of the cell wall. β-Lactams include penicillin derivatives, cephalosporins, carbapenems, and monobactams that interfere with bacterial cell wall synthesis. β-Lactams inhibit the working of PBPs that catalyze the transpeptidation process during peptidoglycan synthesis, thereby preventing crosslinks that form tightly bound cell wall structures. The common mechanisms of β-lactam resistance are alterations of the target sites (gene mutations of the PBPs) (Tang et al., 2014). When a mutation arises, it results in the loss of affinity between β-lactam antibiotics and their target PBPs. Consequently, the antibiotics become incapable of binding to their targets, thus urging bacterial resistance. (Miyachiro et al., 2019). Another example is the resistance to aminoglycosides. Aminoglycosides bind to the decoding center aminoacyl-tRNA recognition site (A-site) on the ribosomal 16S rRNA of the bacteria, thereby inhibiting protein synthesis and thus displaying bactericidal activity. Resistance development related to their use is due to an acquired inactivation of the binding of the aminoglycosides due to the presence of enzymes such as the 16S rRNA methylase that methylates the 16S rRNA target, which can lead to a decrease or loss of affinity of the antibiotic for its target (Poirel et al., 2018).

1.3.2 Resistance to antibiotics by inactivation enzyme

Enzyme-catalyzed modifications of antibiotics are the main mechanisms of antibiotic resistance. At present, thousands of enzymes that can degrade and modify different classes of antibiotics have been identified, including those that modify β-lactams, aminoglycosides, phenicols and macrolides (Mcphee et al., 2009). For example, the most clinically relevant aminoglycosides commonly used to treat infections caused by Gram-negative pathogen are gentamicin (GEN), amikacin (AMK), and tobramycin (TOB) (Partridge et al., 2015). It is reported that more than 100 aminoglycoside-modifying enzymes (AMEs) have been found and are classified into three groups: acetyltransferases, nucleotidyltransferases and phosphotransferases. These AMEs decrease the binding ability of the antibiotic for its target and lead to an ineffective antibacterial ability (Ramirez et al., 2010). Another example is the resistance to carbapenems. The main mechanism of *Enterobacteriaceae* resistance to carbapenem antibiotics is the production of enzymes that hydrolyze carbapenems and the so-called carbapenemase-producing *Enterobacteriaceae* have thus a selective advantage. The β-lactamases can destroy most β-lactams such as penicillin and cephalosporin (Figure 1-8). They are usually produced by, amongst others, resistant *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Sköld et al., 2011; Sawa et al., 2020).

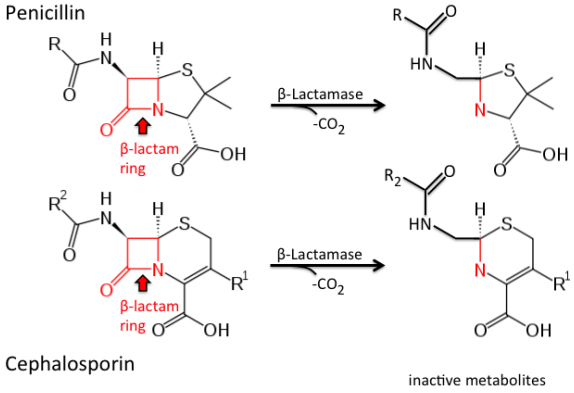


Figure 1-7. Core structures for penicillins & cephalosporin antibiotics & hydrolysis by beta-lactamase. Penicillins & cephalosporins share a common four-atom β-lactam ring. Beta-lactamases are a family of enzymes produced by some gram-negative bacteria that provide a resistance to beta-lactam drugs by breaking the ring open by hydrolysis, which eliminates the molecule's antibacterial actions（https://tmedweb.tulane.edu/pharmwiki/doku.php/beta_lactam_working_rough_draft_-_not_ready_for_prime_time）.

1.3.3 Metabolic alteration or auxotrophy

The bacterial metabolism has been confirmed to be a potential target for antibiotic resistance, but metabolic dysregulation is not commonly recognized as a prominent mechanism of antibiotic resistance (Zhang et al., 2022). However, it has been reported that gene mutations affecting core genes in certain metabolic pathways can lead to antibiotic resistance. For example, the sucA gene (encoding the 2-oxoglutarate dehydrogenase enzyme) involved in the tricarboxylic acid cycle has been found to be widely present in the genome of clinically pathogenic *E. coli* (Zampieri et al., 2021). A mutation in the *suc*A gene reduces basal respiration by inhibiting the activity of the tricarboxylic acid cycle. Zampieri et al. has seen that this led to a reduced bactericidal effect of the antibiotic, and finally to antibiotic resistance. This shows that antibiotic efficacy can be linked to an organism's metabolic state (Zampieri et al., 2021).

Microbial communities consist of cells with all kinds of metabolic capabilities, often including auxotrophs that lack essential metabolic pathways. Auxotrophs can hence only persist in communities where these essential metabolites are consistently available for them to use (Yu et al., 2022). For example, during the process of bacterial folic acid metabolism, sulfonamides possess a similar structure to that of p-aminobenzoic acid (PABA), it can bind with the active site of dihydropteroate synthase (DHPS) by competing with PABA to inhibit the activity of dihydrofolate synthase and prevent folic acid metabolism. DHPS is an essential enzyme in the bacterial folic acid metabolism pathway. Its primary role is to catalyze the formation of dihydropteroate, a crucial precursor in the synthesis of folic acid within bacterial cells. Since folic acid is the precursor of nucleic acid

synthesis, the lack of folic acid will hinder the synthesis of nucleic acid and inhibit the growth and reproduction of bacteria. However, bacteria can also weaken the inhibitory effect of sulfonamide on folic acid metabolism through metabolic enhancement and can also obtain folic acid from extracellular sources in an auxotrophic method to maintain normal metabolism (Figure 1-8) (modified from Zhang et al., 2022).

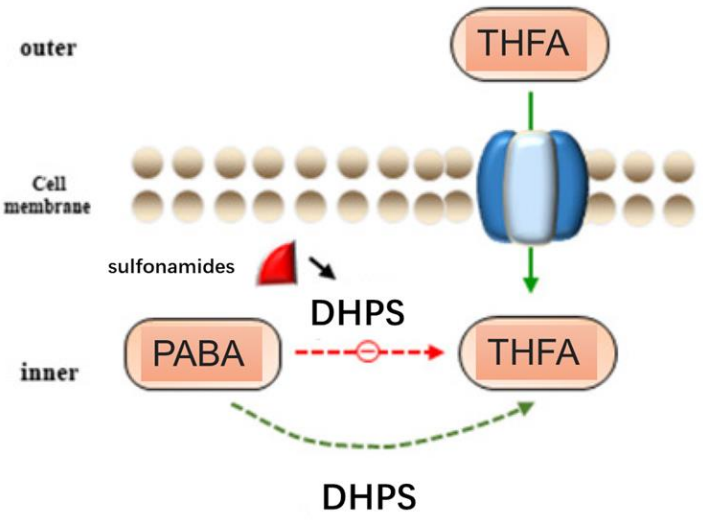


Figure 1-8. The mechanism of metabolic alteration or auxotrophy. tetrahydrofolic acid (THFA), dihydropteroate synthase (DHPS), p-aminobenzoic acid (PABA).

1.3.4 Efflux pumps

Efflux pumps are ubiquitous transport proteins distributed in the plasma membrane of bacteria, archaea and eukaryotes (Tanaka et al., 2013). Bacteria can use efflux pumps as a natural defense mechanism as they are able to expel various toxic compounds from the environment (Henderson et al., 2021). Efflux pumps enable bacteria to survive for a period of time, increasing the likelihood of spontaneous mutations leading to the development of high levels of resistance to some antibiotics (Ebbensgaard et al., 2020). Efflux pumps have been implicated as a kind of effective resistance mechanism in bacterial pathogens to a variety of antibiotics clinically used to treat infections (Blanco et al., 2016). When overexpressed, efflux pumps can confer high levels of resistance to commonly used clinical antibiotics. Some efflux pumps have narrow substrate specificity such as *tet* pumps, but many transport a variety of structurally distinct substrates and are known as multidrug-resistant (MDR) efflux pumps (Blair et al., 2015).

There are several important efflux transporters families taking part of these MDR efflux pumps: Resistance Nodule and Cell Division (RND), especially important for bacteria; Master Facilitator Superfamily (MFS); Multidrug and Toxic Compound Extrusion (MATE); small multidrug resistance (SMR); and the ATP-binding cassette (ABC) superfamily or family (Hernando-Amado et al., 2016). ABC efflux pumps utilize ATP hydrolysis to consume energy and eliminate substrates, whereas MATE, MFS, RND, and SMR pumps transport sodium and hydrogen out of membranes by utilizing the proton motive force (PMF) as an energy source (Kourtesi et al., 2013). Bacteria overexpressing efflux pumps have been isolated from patients since the 1990s, including *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Everett et al.,1996; Pumbwe et al., 2000; Kosmidis et al.,2012). Understanding the mechanism of overexpression is important because it is a common resistance mechanism in clinical isolates, and a thorough understanding of this mechanism could lead to the design of new treatments to prevent the production of efflux pump proteins.

1.3.5 Reduced permeability

In Gram-negative bacteria, the cell wall is mainly composed of proteins and lipopolysaccharides, where hydrophilic antibiotics diffuse across the outer membrane via the outer membrane porin (Omp) proteins (Chevalier et al., 2017). Each kind of bacteria produces specific porins such as OmpF, OmpC, and OmpE, and one or more deletions in them or damage to the Omps is one of the sources of bacterial resistance (McPhee et al., 2009). For example, the absence of the OprD porin on the outer membrane of cells results in low efficacy or weakness of many broad-spectrum antimicrobials against *Pseudomonas aeruginosa*, and the inability of antimicrobials to enter cells leads to natural resistance to antibiotics (Nadeem et al., 2020).

1.4 Current situation of bacterial resistance in milk

1.4.1 Current situation of *Staphylococcus*

Staphylococcus spp. is part of the commensal microbiota on the skin and in nostrils in healthy humans and animals, but they may also cause infections, resulting in abscesses and septicemia, and potentially also leading to bacterial diseases (Ikwap et al., 2021). As such, *Staphylococcus* spp. forms a big problem in cattle, since it can colonize the udder of dairy cows, leading to the occurrence of mastitis (Sasidharan et al., 2011). Clinical mastitis can be diagnosed by recognizing specific visible symptoms, which may include observable signs like redness in the udder, increased warmth, noticeable swelling, sensitivity to touch, the presence of milk clots, alterations in the color and texture of the milk. Frequently observed indications also encompass fever (with a temperature exceeding 39.5 °C) and a reduction in appetite (Sharun et al., 2021). According to the data from the National Bureau of Statistics of China for the year 2022 (source: https://data.stats.gov.cn), China is home to approximately 102.16 million cattle, with an estimated mastitis prevalence rate of approximately 60-70%. The bacteria are transmitted to dairy cows from the environment, and when adequate sanitation is lacking during milking, they are transmitted along the dairy production chain (Silva et al., 2022). Humans can then consume these bacteria, which can cause

disease. *Staphylococcus spp.* is of the most important pathogens affecting human health, and *staphylococcal* poisoning is the most common type of foodborne disease (Hennekinne et al., 2012). Although *staphylococci* in dairy products are nonpathogenic, they can become opportunistic and cause nosocomial infections (Irlinger et al., 2008). It is reported that the multidrug resistance rate of *staphylococci* isolated from cows' milk ranges from 18% to 34% in Kenya (Mbindyo et al., 2021). Deaths from AMR in Africa are likely to be greater than in the rest of the world because surveillance data is scarce. In industrialized countries, these data are available at the regional, national and international levels. For example, the European Antimicrobial Resistance Surveillance System (EARSS) provides a database with information on AMRs (De Kraker et al., 2013). However, also in the developing countries, Staphylococcus remains a problem. A growing number of studies reported that *Staphylococcus aureus* has developed antibiotic resistance and evolved from single-drug resistance to multidrug resistance (MDR), making it increasingly difficult to address antibiotic resistance (Gomes et al., 2016). *Staphylococcus aureus* was responsible for approximately 119,000 morbidities and almost 20,000 deaths in the United States in 2007 (Kourtis et al., 2019). Methicillin-resistant *S. aureus* (MRSA) bacteria are among the most pathogenic *S. aureus*, and they exhibit resistance levels to multiple antibiotics, particularly penicillin, aminoglycosides, macrolides, tetracyclines, and fluoroquinolones (Hasanpour et al., 2017).

1.4.2 Current situation of *Escherichia coli*

Escherichia coli is a Gram-negative rod-shaped commensal bacterium in the human intestine and it is also a major causative agent of several infection diseases. *Escherichia coli* is also a very important environmental pathogen causing mastitis in dairy cows. In a study by Liu et al., a total of 1920 retail milk samples were obtained from grocery stores in California in 2017 (Liu et al., 2020). Among the 1920 milk samples, 95 *E. coli* strains were isolated, 80 (84%) had at least one antibiotic-resistant phenotype and 34 (36%) showed multidrug resistance (≥2 antibiotic-resistant phenotypes). Ceftazidime resistance was the most prevalent phenotype in this cohort (n=57; 60%), followed by resistance against amoxicillin (n=24; 25%), tetracycline (n=24; 25%), and streptomycin (n=22; 23%). The antibiotic-resistant *E. coli* also emerges in low-income countries. A recent study evaluated the risk factors related to the carriage of antibiotic-resistant *E. coli* in northern Tanzania and found that direct transmission of microorganisms in raw milk was a major predictor of AMR prevalence, emphasizing the role of raw milk in transmitting antibiotic-resistant *E. coli* (Caudell et al., 2018). Liu et al. collected 195 raw milk samples from dairy farms in northern China. Among the samples, 67 *E. coli* strains were isolated. The prevalence of β-lactamase-encoding genes was 34% in those 67 *E. coli* isolates and 45% in 40 β-lactam-resistant *E. coli* isolates (Liu et al., 2021).

1.4.3 Current situation of *Bacillus cereus*

Bacillus cereus is a Gram-positive, endospore-forming, foodborne pathogen that is widely distributed in the natural environment, commonly found in foods

especially dairy products, and they even persist in host epithelial cells (Rigourd et al., 2018; Abdeen et al., 2020). Foodborne outbreaks involving *B. cereus* often occur through dairy products in China (Gao et al., 2018; Zhao et al., 2020). Precautionary measures have been taken in the last century to manage contamination of dairy products, but outbreaks of *B. cereus* contamination still occur in a variety of dairy products around the world (Rossi et al., 2018; Yusuf et al., 2018; Pedonese et al., 2019). Effective antibiotic treatment is considered as the main therapy to eliminate *B. cereus* infection, so it is necessary to study the antimicrobial susceptibility of *B. cereus*. In a recent study, *B. cereus* strains isolated from pasteurized milk in China were resistant to β-lactam antibiotics and rifampicin, but were sensitive to quinolones, aminoglycosides, and macrolides (Gao et al., 2018). It is reported that the *B. cereus* isolated from dairy farms and dairy products were generally resistant to β-lactam antibiotics such as ampicillin (98%), oxacillin (92%), penicillin (100%), amoxicillin (100%), and cefepime (100%) (Owusu-Kwarteng et al., 2017). Torkar et al. (2016) considered vancomycin as one of the most appropriate antibiotic options to combat *B. cereus* infections (Torkar et al., 2016), but Gao et al. found a fraction of the isolates detected (approximately 13%) that were not susceptible to vancomycin (Gao et al., 2018), revealing a potentially high risk of *B. cereus* to public health and the dairy industry.

1.5 Measures against bacterial resistance

1.5.1 One health strategy for the prevention and control of antibiotic resistance

Deaths due to antibiotic resistance are estimated around 700,000 people worldwide each year (European Comission, 2017). To address this problem, it is often incorporated into “The One Health Model”. “One Health” is defined as a multidisciplinary effort - locally, nationally and globally - to achieve optimal health for humans, animals and the environment through policy, research, education and practice (Gronvall et al., 2014). In 2008, several organizations including the FAO, the WOAH and the WHO emphasized the importance of ecosystems in the One Health concept (FAO/OIE/WHO, 2008). In general, infectious diseases, including zoonoses, are the major focus and target of the One Health approach. AMR is directly linked to such diseases and it has been recognized as one of the major problems deserving a One Health approach (Van Puyvelde et al., 2018). To address the crisis of antibiotic contamination and antibiotic resistance globally, the WHO provided the framework for national action plans in 2015, developed policies and regulations to combat ARB and antibiotic contamination. For example, Canada participates in global efforts to combat antibiotic contamination and ARB, and committed to address antimicrobial resistance and antimicrobial use through its own domestic program. The program focuses on four main topics: (1) Surveillance: While the Canadian surveillance systems are producing useful and reliable data on AMR and antibiotic use, there is still a lack of information in certain communities and the robust integrated surveillance systems are needed to fully understand AMR and antibiotic use in Canada. (2) Infection prevention and control: To curb the spread

of resistant microorganisms and reduce AMRs and antibiotic use, standardized infection prevention and control approaches, programs and policies must be developed. (3) Stewardship: To develop programs and policies to raise awareness to reduce inappropriate prescribing and use of antibiotics in humans and animals, and to protect the effectiveness of new and existing antibiotics. (4) Innovation: Responses to AMR must be evidence-based and require increased knowledge, innovative tools and collaborative methods to better understand resistance and develop new treatments and strategies (Public Health Agency of Canada, 2017). Europe has been at the forefront of addressing antibiotic contamination and the antibiotic resistance crisis and implemented the "EU One Health Action Plan against AMR" in June 2017. The main objectives of their program are: (1) To make the EU a best practice region, (2) To boost research, development and innovation, and (3) To shape the global agenda (Kraemer et al., 2019).

1.5.2 Control the use of veterinary antibiotics and maintain the ecological balance of microorganisms

For decades, many antibiotics were given to food-producing animals for production purposes, such as growth promotion. However, now, these practices are no longer permitted. Countries such as Canada, India, and European member states have implemented and enhanced surveillance systems to detect new emerging threats and monitor changes in antibiotic usage and the prevalence of ARB in agricultural settings, particularly in animal production, with a focus on promoting appropriate use of antibiotics in veterinary medicine. Since 1999, the EU no longer permits the use of antibiotics that are medically important to humans as additives to animal feed for economic purposes of promoting growth. Since the 1st of January 2006, the growth promotion uses of the remaining antibiotics (non-medically important) were forbidden (European Commission, 2003). The European Parliament voted in 2019 to forbid antibiotic use for disease prevention, effective from the 28th of January, 2022 (Wallinga et al., 2022). Canada has implemented a strong regulatory framework for veterinary drugs and medicated feeds since February 2018, including adding medically important antimicrobials (MIAs) to the Prescription Drug List (PDL) to make sure they are sold by prescription only, and removing growth promotion claims from MIA drug labels (CCDR, 2017). China also passed the legislation, effective from November 2020, banning antibiotics as feed additives to promote growth (Wen et al., 2022). In the US, the Food and Drug Administration (FDA) made the use of antibiotics as mere growth promotors illegal in 2017 (Wallinga et al., 2022). Nevertheless, the FDA clearly defines antibiotics used for disease prevention as therapeutic drugs, even when used in animals without any disease or specific pathogen infection. At least 13 medically important antibiotics are FDA-approved for use in feeds for disease prevention with no clear time ("duration") limit, meaning that animal populations may be exposed to them on a near-continuous basis (Hyun et al., 2021). Developing countries should examine and build on the experiences of developed countries and obtain scientific expertise from these countries to reduce antibiotic consumption in livestock animals and aquaculture while maintaining their productivity. In addition, it is critical to promote and

ensure sustainable agriculture so that developing countries are not dependent on antibiotics, and this to achieve the United Nations Sustainable Development Goals by 2030.

1.5.3 Improve sanitation conditions of husbandry and reduce cross-contamination

Unprecedented global food demands lead to farmers relying on antibiotics to produce large amounts of animal protein at low cost. Improving animal welfare and health has the potential to reduce overreliance on antibiotics without compromising productivity and costs (Founou et al., 2016). One potential solution could involve applying tightly regulated extensive farming practices that prioritize animal health and welfare, while promoting and implementing the use of low levels of chemicals. This method aims to limit the development of infectious diseases and reduce reliance on antibiotics. Practices such as all-in-all-out management and extensive free-range systems could be encouraged instead of intensive farming methods. Free-range systems typically involve lower animal densities compared to intensive farming systems. The reduced crowding and lower stress levels in animals can contribute to better overall health and a lower risk of disease transmission, which may decrease the need for antibiotic use. Organic farms have become very popular in recent years, both to meet global food needs and to reduce the widespread use of antibiotics (Prabhakar et al., 2010). For example, Österberg et al. reported that the prevalence of antibiotic resistance *E. coli* isolated from organic pig farms was significantly lower than that from conventional pig farms in four European countries (Ligon et al., 2004). It is also reported that the prevalence of MRSA in the free-range production type herds is lower than that in the conventional indoor production herds (20.2% to 89.2%) (European Food Safety Authority, 2021).

1.5.4 Examine the use of antibiotic replacements

Using natural antimicrobials is one of the most effective methods to reduce antibiotic resistance. Studies to discover potential alternatives, such as pre- or probiotics, have progressed in recent years. For example, many probiotics are used to treat and control *Staphylococcus* infections. The probiotic *Lactobacillus casei* (BL23) significantly decreases mammary glands inflammation during *S. aureus* infection by inhibiting the expression of *S. aureus*-induced pro-inflammatory cytokines (IL8, IL6, TNF-α, IL1β, and IL1α). This causes a potent anti-inflammatory effect against *S. aureus* infection in bovine mammary epithelial cells (Souza et al., 2018). It is reported that the cleaning solutions and probiotic agents were administered directly to the udder teats of dairy cows by massage movements to the teat apex area twice a day, directly after milking. Notably, the control group, which did not receive probiotics, experienced a substantial rise in the number of diseased animals within one month (Zhumakayeva et al., 2023). Some antioxidants such as polyphenols, vitamins and carotenoids have good antibacterial and anti-inflammatory activities (Naqvi et al., 2019; Abd EI-Ghany et al., 2020). Awan et al. found that chloroform extract of cumin and turmeric exhibited significant antibacterial activity against *Serratia marcescens* and *P. aeruginosa* (Awan et al., 2013). Nweze and Eze reported that the ethanolic extract

of lamiaceae leaves mixed with ampicillin had a synergistic influence on *E. coli* and *Candida albicans* (Nweze et al., 2009).

1.6 Mechanisms of heteroresistance

The broadest definition of heteroresistance (HR) is the presence of a heterogeneous bacterial population with one or a few subpopulations that exhibit higher levels of antibiotic resistance compared to the main population (El-Halfawy et al., 2015). The first reported use of the term "heteroresistance" occurred in 1970 (Kayser et al., 1970). Increased antibiotic resistance may be due to mutations or gene duplications in key resistance genes or regulatory systems. Long-term infection may lead to instability of bacterial genomic DNA, which may lead to HR.

1.6.1 Examples of heteroresistance

For example, Chambers et al. (1985) showed that an increased production of PBP2a, encoded by the *mecA* gene, was responsible for an increased methicillin resistance in a subset of a *S. aureus* population, thus leading to HR in a population (Chambers et al., 1985). However, regulatory systems can also lead to HR towards β-lactams. For example, the inactivation of transcriptional regulators, such as Sar (Piriz et al., 1996) and the Sigma B operon (Wu et al., 1996), are a possible cause for MRSA HR. However, Sigma B contributed to methicillin resistance but not to HR in *S. epidermidis.* The inactivation of the anti-Sigma B factor RsbW converts HR to a uniform high-level resistance (Knobloch et al., 2005). Among *P. aeruginosa* carbapenem-HR isolates, a stable resistant subpopulation showed an upregulation of efflux-related genes and increased membranous permeability to carbapenems due to the reduced expression of the porin-encoding gene *oprD* (He et al., 2018).

HR has also been found towards glycopeptides**.** An example of a glycopeptide antibiotic is vancomycin. HR to glycopeptide antibiotics is not directly linked to a specific mechanism. Several studies reported an increased incidence of regulatory gene mutations in HR populations. The *agr* (accessory gene regulator) was dysfunctional in 58% HR vancomycin intermediate *S. aureus* (hVISA) isolates but only in 21% of MRSA strains (Arigaya et al., 2011). It seems that *agr* dysfunction leads to the development of vancomycin HR of *S. aureus* clinical isolates. For example, compared with vancomycin sensitive MRSA, 13 of 38 (34%) hVISA isolates had at least 1 non-synonymous mutation: 6 in *vraSR*, 7 in *walRK*, and 2 in *rpoB* (Yamakawa et al., 2012). Mutations in the *vraS* gene lead to upregulation of the VraSR two-component system and conversion to the hVISA phenotype (Katayama et al., 2009). Various mutations within an essential *walKR* two-component regulatory site involved in the control of cell wall metabolism, increase resistance to vancomycin and daptomycin in several hVISA strains (Howden et al., 2011). Independent novel mutations in the *vanR*, *vanS*, *vanH*, *vanA*, *vanX*, and *vanY* genes that occur during sustained exposure to antibiotics can cause HR in vancomycin-resistant *Enterococcus* strains (Khan et al., 2008; Park et al., 2008).

In several studies, the resistance mechanism of subpopulations with stable HR involved efflux and/or influx of antibiotics. Resistance is associated with an increased expression of efflux pumps in colistin HR *A. baumannii* isolates (Machado et al., 2018). HR to polymyxin B in *B. cereus* depends on differences in putrescine and *YceI* secretion levels that are differentially expressed in different subpopulations (l-Halfawy et al., 2013). Gene amplification-driven HR was first reported in a study of colistin resistance in *S. typhimurium* (Hjort et al., 2016). The results showed that during sub-MIC selection (i.e., concentrations that are lower than the MIC, which can contribute to antibiotic resistance for colistin resistance) mutants exhibited an HR phenotype due to the amplification of the gene containing *pmrD*.

1.6.2 Detection methods of HR

The Population Analysis Profiling (PAP) method is considered the golden standard for determining HR. In this method, bacterial populations are subjected to antibiotic concentration gradients (on plates or in liquid media), and bacterial growth at each concentration is quantified. PAP is usually performed using a standard MIC assay format with 2-fold increments of the antibiotic, and using the spread plate method for colony-forming unit (CFU) enumeration (Figure 1-9).

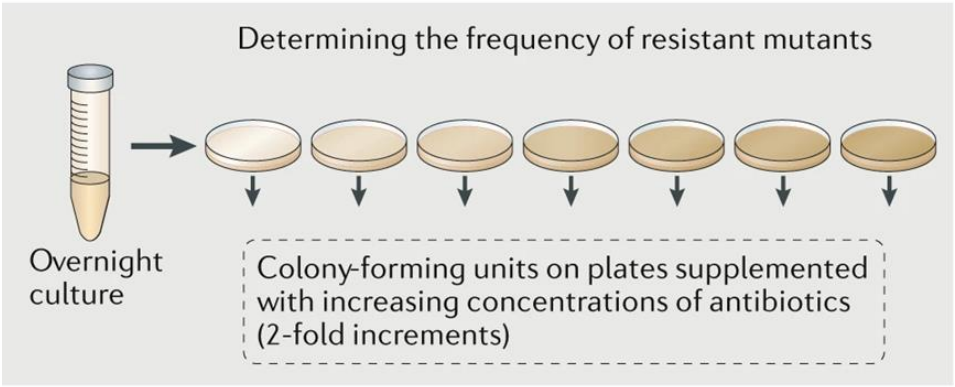


Figure 1-9. Population analysis profile (PAP) test to identify HR. The principle of the PAP test is shown in the figure. Briefly a defined number of cells (typically 10^2 to 10^8 cells) are spread on agar plates with different concentrations of the antibiotic, and the number of colonies formed are counted after 1 day to determine the frequency of resistant cells and their level of resistance. One frequently accepted definition of HR is growth of a resistant subpopulation at antibiotic concentrations at least eightfold above the highest antibiotic concentration that does not affect growth of the main sensitive population (Andersson et al., 2019).

The disc diffusion test and Etest strips have also been used to detect HR and are recommended for traditional *in vitro* susceptibility testing (Pelaez et al., 2008; Superti et al., 2009; Tato et al., 2010; Lee et al., 2011). Compared with PAP, the lack of standard guidelines hinders the use of the Etest and the disc diffusion

assays to detect HR. These and other methods all show poor specificity and poor sensitivity, as indicated by the high frequency of false positive and false negative samples (Lo-Ten-Foe et al., 2007; Van Hal et al., 2011). A clear sign of HR is the growth of distinct colonies growing within the clear zone of inhibition as determined by the Etest or the disc diffusion assay. However, as mentioned earlier, many reports set cut-off concentrations or zone-inhibition diameters to determine the heterogeneity of bacterial population responses to antibiotics, but these cut-offs do not adequately describe population-wide behavior.

Therefore, by simultaneously investigating both milk and fecal samples, our research aimed to provide a comprehensive evaluation of the broader impacts of cephalosporin administration on the overall microbial ecology within dairy cows. This unique dual-sampling approach allowed us to explore the potential transmission and alterations in microbial populations originating from the gut (as represented by feces) to the udder (as represented by milk). Our primary focus was to gain insights into the dynamics of antibiotic resistance. Antibiotic-resistant bacteria play a central role in the ineffectiveness of antibiotic treatments. However, it's important to note that the mere identification of bacteria as 'sensitive' to antibiotics does not guarantee the success of antibiotic therapy in managing bacterial infections. This complexity arises from the population's ability to exhibit phenotypic heterogeneity. Within this context, heteroresistance emerges as a critical intermediate stage in the progression toward full antibiotic resistance. Understanding the intricacies of heteroresistance is essential for developing more effective strategies to combat bacterial infections and to ensure the judicious use of antibiotics in veterinary medicine.

1.7 References

Aarestrup F. M. (2005). Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. Basic & clinical pharmacology & toxicology, 96(4), 271–281.

Abdeen, E. E., Hussien, H., Hadad, G. A. E., & Mousa, W. S. (2020). Prevalence of Virulence Determinants among Bacillus cereus Isolated from Milk Products with Potential Public Health Concern. Pakistan journal of biological sciences: PJBS, 23(3), 206–212.

Abd El-Ghany W. A. (2020). Paraprobiotics and postbiotics: Contemporary and promising natural antibiotics alternatives and their applications in the poultry field. Open veterinary journal, 10(3), 323–330.

Acar, J. F., & Moulin, G. (2006). Antimicrobial resistance at farm level. Revue scientifique et technique (International Office of Epizootics), 25(2), 775–792.

Akram, R., Amin, A., Hashmi, M.Z., Wahid, A., Mubeen, M., Hammad, H.M., Fahad, S., & Nasim, W. (2017). Fate of Antibiotics in Soil.

Andersson, D. I., and Hughes, D. (2014). Microbiological effects of sub-lethal levels of antibiotics. Nat. Rev. Microbiol. 12. 465–478.

Andersson, D. I., Nicoloff, H., & Hjort, K. (2019). Mechanisms and clinical relevance of bacterial heteroresistance. Nature reviews. Microbiology, 17(8), 479–496.

Aslam, B., Khurshid, M., Arshad, M. I., Muzammil, S., Rasool, M., Yasmeen, N., Shah, T., Chaudhry, T. H., Rasool, M. H., Shahid, A., Xueshan, X., & Baloch, Z. (2021). Antibiotic Resistance: One Health One World Outlook. Frontiers in cellular and infection microbiology, 11, 771510.

Awan, U. A., Andleeb, S., Kiyani, A., Zafar, A., Shafique, I., Riaz, N., Azhar, M. T., & Uddin, H. (2013). Antibacterial screening of traditional herbal plants and standard antibiotics against some human bacterial pathogens. Pakistan journal of pharmaceutical sciences, 26(6), 1109–1116.

Bailey, A. L., Armstrong, T., Dwivedi, H. P., Denys, G. A., Hindler, J., Campeau, S., Traczewski, M., Humphries, R., & Burnham, C. A. (2018). Multicenter Evaluation of the Etest Gradient Diffusion Method for Ceftolozane-Tazobactam Susceptibility Testing of Enterobacteriaceae and Pseudomonas aeruginosa. Journal of clinical microbiology, 56(9), e00717-18.

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. Nature reviews. Microbiology, 13(1), 42–51.

Blanco, P., Hernando-Amado, S., Reales-Calderon, J. A., Corona, F., Lira, F., Alcalde-Rico, M., Bernardini, A., Sanchez, M. B., & Martinez, J. L. (2016). Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants. Microorganisms, 4(1), 14.

Broom L. J. (2017). The sub-inhibitory theory for antibiotic growth promoters. Poultry science, 96(9), 3104–3108.

Cabello, F. C., Godfrey, H. P., Buschmann, A. H., & Dölz, H. J. (2016). Aquaculture as yet another environmental gateway to the development and globalisation of antimicrobial resistance. The Lancet. Infectious diseases, 16(7), e127–e133.

Cabello, F. C., Godfrey, H. P., Tomova, A., Ivanova, L., Dölz, H., Millanao, A., & Buschmann, A. H. (2013). Antimicrobial use in aquaculture re-examined: its relevance to antimicrobial resistance and to animal and human health. Environmental microbiology, 15(7), 1917–1942.

Cafiso, V., Bertuccio, T., Spina, D., Purrello, S., Campanile, F., Di Pietro, C., Purrello, M., & Stefani, S. (2012). Modulating activity of vancomycin and daptomycin on the expression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. PloS one, 7(1), e29573.

Callaway, T. R., Lillehoj, H., Chuanchuen, R., & Gay, C. G. (2021). Alternatives to Antibiotics: A Symposium on the Challenges and Solutions for Animal Health and Production. Antibiotics (Basel, Switzerland), 10(5), 471.

Cameron, A., & McAllister, T. A. (2016). Antimicrobial usage and resistance in beef production. Journal of animal science and biotechnology, 7, 68.

Caudell, M. A., Mair, C., Subbiah, M., Matthews, L., Quinlan, R. J., Quinlan, M. B., Zadoks, R., Keyyu, J., & Call, D. R. (2018). Identification of risk factors associated with carriage of resistant Escherichia coli in three culturally diverse ethnic groups in Tanzania: a biological and socioeconomic analysis. The Lancet. Planetary health, 2(11), e489–e497.

CCDR. (2017). Antimicrobial stewardship and veterinary drug regulation. Volume 43-11: Antimicrobial resistance and One Health.

Chambers, H. F., Hartman, B. J., & Tomasz, A. (1985). Increased amounts of a novel penicillin-binding protein in a strain of methicillin-resistant Staphylococcus aureus exposed to nafcillin. The Journal of clinical investigation, 76(1), 325–331.

Chen, J., Ying, G. G., & Deng, W. J. (2019). Antibiotic Residues in Food: Extraction, Analysis, and Human Health Concerns. Journal of agricultural and food chemistry, 67(27), 7569–7586.

Chevalier, S., Bouffartigues, E., Bodilis, J., Maillot, O., Lesouhaitier, O., Feuilloley, M. G. J., Orange, N., Dufour, A., & Cornelis, P. (2017). Structure, function and regulation of Pseudomonas aeruginosa porins. FEMS microbiology reviews, 41(5), 698–722.

Coetzee, J., Corcoran, C., Prentice, E., Moodley, M., Mendelson, M., Poirel, L., Nordmann, P., & Brink, A. J. (2016). Emergence of plasmid-mediated colistin resistance (MCR-1) among Escherichia coli isolated from South African patients. South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde, 106(5), 35–36.

Cycoń, M., Mrozik, A., & Piotrowska-Seget, Z. (2019). Antibiotics in the Soil Environment-Degradation and Their Impact on Microbial Activity and Diversity. Frontiers in microbiology, 10, 338.

Dantas, G., & Sommer, M. (2014). How to Fight Back Against Antibiotic Resistance. American Scientist, 102(1), 42-51.

de Kraker, M. E., Jarlier, V., Monen, J. C., Heuer, O. E., van de Sande, N., & Grundmann, H. (2013). The changing epidemiology of bacteraemias in Europe: trends from the European Antimicrobial Resistance Surveillance System. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 19(9), 860–868.

Drury, B., Scott, J., Rosi-Marshall, E. J., & Kelly, J. J. (2013). Triclosan exposure increases triclosan resistance and influences taxonomic composition of benthic bacterial communities. Environmental science & technology, 47(15), 8923–8930.

Ebbensgaard, A. E., Løbner-Olesen, A., & Frimodt-Møller, J. (2020). The Role of Efflux Pumps in the Transition from Low-Level to Clinical Antibiotic Resistance. Antibiotics (Basel, Switzerland), 9(12), 855.

Economou, V., & Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. Infection and drug resistance, 8, 49–61.

El-Halfawy, O. M., & Valvano, M. A. (2015). Antimicrobial heteroresistance: an emerging field in need of clarity. Clinical microbiology reviews, 28(1), 191–207.

El-Halfawy, O. M., & Valvano, M. A. (2013). Chemical communication of antibiotic resistance by a highly resistant subpopulation of bacterial cells. PloS one, 8(7), e68874.

EUCAST. (2021) "Antimicrobial susceptibility testing: EUCAST disk diffusion method". EUCAST. Retrieved March 16.

European Comission. (2017). A European One Health Action Plan against Antimicrobial Resistance (AMR); European Comission: Brussels, Belgium,.

European Commission. (2003). Regulation (ec) no 1831/2003 of the european parliament and of the council of 22 september 2003 on additives for use in animal nutrition. Available: https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32003R183126 April 2021.

European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Dieases (ESCMID) (2000). EUCAST Definitive Document E. Def 1.2, May 2000: Terminology relating to methods for the determination of susceptibility of bacteria to antimicrobial agents. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 6(9), 503–508.

European Food Safety Authority; European Centre for Disease Prevention and Control. (2021) The European Union Summary Report on Antimicrobial

Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. EFSA J. Apr 12;19(4): e06490.

European Union. (2005). Ban on antibiotics as growth promoters in animal feed enters into efect Available: http://europa.eu/rapid/press-release_IP-05-1687_en.htm 26 April 2021.

Everett, M. J., Jin, Y. F., Ricci, V., & Piddock, L. J. (1996). Contributions of individual mechanisms to fluoroquinolone resistance in 36 Escherichia coli strains isolated from humans and animals. Antimicrobial agents and chemotherapy, 40(10), 2380–2386.

FAO/OIE/WHO (Food and Agriculture Organization of the United Nations/World Organization for Animal Health/World Health Organization). (2006). Antimicrobial Use in Aquaculture and Antimicrobial Resistance. Available: http://www.who.int/topics/foodborne_diseases/aquaculture_rep_13_16june2006%20.pdf [accessed 18 April 2013].

FAO/OIE/WHO (Food and Agriculture Organization of the United Nations/World Organization for Animal Health/World Health Organization). (2008). A Strategic Framework for Reducing Risks of Infectious Diseases at the Animal–Human–Ecosystems Interface. Available: https://www.fao.org/3/aj137e/aj137e00.pdf.

Founou, L. L., Founou, R. C., & Essack, S. Y. (2016). Antibiotic Resistance in the Food Chain: A Developing Country-Perspective. Frontiers in microbiology, 7, 1881.

Gao, T., Ding, Y., Wu, Q., Wang, J., Zhang, J., Yu, S., Yu, P., Liu, C., Kong, L., Feng, Z., Chen, M., Wu, S., Zeng, H., & Wu, H. (2018). Prevalence, Virulence Genes, Antimicrobial Susceptibility, and Genetic Diversity of Bacillus cereus Isolated From Pasteurized Milk in China. Frontiers in microbiology, 9, 533.

Godič Torkar, K., Bedenić, B., & Plečko, V. (2016). Antimicrobial susceptibility and the in vitro postantibiotic effects of vancomycin and ciprofloxacin against Bacillus cereus isolates. Journal of chemotherapy (Florence, Italy), 28(3), 151–158.

Gomes, F., & Henriques, M. (2016). Control of Bovine Mastitis: Old and Recent Therapeutic Approaches. Current microbiology, 72(4), 377–382.

Grada, A., & Bunick, C. G. (2021). Spectrum of Antibiotic Activity and Its Relevance to the Microbiome. JAMA network open, 4(4), e215357.

Grenni, P., Ancona, V., & Caracciolo, A.B. (2018). Ecological effects of antibiotics on natural ecosystems: A review. Microchemical Journal, 136, 25-39.

Gronvall, G., Boddie, C., Knutsson, R., & Colby, M. (2014). One health security: an important component of the global health security agenda. Biosecurity and bioterrorism: biodefense strategy, practice, and science, 12(5), 221–224.

Harigaya, Y., Ngo, D., Lesse, A. J., Huang, V., & Tsuji, B. T. (2011). Characterization of heterogeneous vancomycin-intermediate resistance, MIC and accessory gene regulator (agr) dysfunction among clinical bloodstream isolates of staphyloccocus aureus. BMC infectious diseases, 11, 287.

Hasanpour Dehkordi, A., Khaji, L., Sakhaei Shahreza, M. H., Mashak, Z., Safarpoor Dehkordi, F., Safaee, Y., Hosseinzadeh, A., Alavi, I., Ghasemi, E., & Rabiei-Faradonbeh, M. (2017). One-year prevalence of antimicrobial susceptibility pattern of methicillin-resistant Staphylococcus aureus recovered from raw meat. Tropical biomedicine, 34(2), 396–404.

Hassan, Y. I., Lahaye, L., Gong, M. M., Peng, J., Gong, J., Liu, S., Gay, C. G., & Yang, C. (2018). Innovative drugs, chemicals, and enzymes within the animal production chain. Veterinary research, 49(1), 71.

He, J., Jia, X., Yang, S., Xu, X., Sun, K., Li, C., Yang, T., & Zhang, L. (2018). Heteroresistance to carbapenems in invasive Pseudomonas aeruginosa infections. International journal of antimicrobial agents, 51(3), 413–421.

Henderson, P. J. F., Maher, C., Elbourne, L. D. H., Eijkelkamp, B. A., Paulsen, I. T., & Hassan, K. A. (2021). Physiological Functions of Bacterial "Multidrug" Efflux Pumps. Chemical reviews, 121(9), 5417–5478.

Hennekinne, J. A., De Buyser, M. L., & Dragacci, S. (2012). Staphylococcus aureus and its food poisoning toxins: characterization and outbreak investigation. FEMS microbiology reviews, 36(4), 815–836.

Hernando-Amado, S., Blanco, P., Alcalde-Rico, M., Corona, F., Reales-Calderón, J. A., Sánchez, M. B., & Martínez, J. L. (2016). Multidrug efflux pumps as main players in intrinsic and acquired resistance to antimicrobials. Drug resistance updates: reviews and commentaries in antimicrobial and anticancer chemotherapy, 28, 13–27.

Hjort, K., Nicoloff, H., & Andersson, D. I. (2016). Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in Salmonella enterica. Molecular microbiology, 102(2), 274–289.

Holmes, A. H., Moore, L. S., Sundsfjord, A., Steinbakk, M., Regmi, S., Karkey, A., Guerin, P. J., & Piddock, L. J. (2016). Understanding the mechanisms and drivers of antimicrobial resistance. Lancet (London, England), 387(10014), 176–187.

Howden, B. P., McEvoy, C. R., Allen, D. L., Chua, K., Gao, W., Harrison, P. F., Bell, J., Coombs, G., Bennett-Wood, V., Porter, J. L., Robins-Browne, R., Davies, J. K., Seemann, T., & Stinear, T. P. (2011). Evolution of multidrug resistance during Staphylococcus aureus infection involves mutation of the essential two component regulator WalKR. PLoS pathogens, 7(11), e1002359.

Hyun D. (2021). FDA must establish limits for all animal antibiotics. Available: https://www.pewtrusts.org/da/research-and-analysis/issue-

briefs/2021/04/fda-must-establish-limits-for-all animal-antibiotics 22 June 2021.

Ikwap, K., Gertzell, E., Hansson, I., Dahlin, L., Selling, K., Magnusson, U., Dione, M., & Jacobson, M. (2021). The presence of antibiotic-resistant Staphylococcus spp. and Escherichia coli in smallholder pig farms in Uganda. BMC veterinary research, 17(1), 31.

Irlinger F. (2008). Safety assessment of dairy microorganisms: coagulase-negative staphylococci. International journal of food microbiology, 126(3), 302–310.

Katayama, Y., Murakami-Kuroda, H., Cui, L., & Hiramatsu, K. (2009). Selection of heterogeneous vancomycin-intermediate Staphylococcus aureus by imipenem. Antimicrobial agents and chemotherapy, 53(8), 3190–3196.

Kayser, F. H., Benner, E. J., & Hoeprich, P. D. (1970). Acquired and native resistance of Staphylococcus aureus to cephalexin and other beta-lactam antibiotics. Applied microbiology, 20(1), 1–5.

Khan, S. A., Sung, K., Layton, S., & Nawaz, M. S. (2008). Heteroresistance to vancomycin and novel point mutations in Tn1546 of Enterococcus faecium ATCC 51559. International journal of antimicrobial agents, 31(1), 27–36.

Knobloch, J. K., Jäger, S., Huck, J., Horstkotte, M. A., & Mack, D. (2005). mecA is not involved in the sigmaB-dependent switch of the expression phenotype of methicillin resistance in Staphylococcus epidermidis. Antimicrobial agents and chemotherapy, 49(3), 1216–1219.

Kocsis, B., Gulyás, D., & Szabó, D. (2022). Emergence and Dissemination of Extraintestinal Pathogenic High-Risk International Clones of Escherichia coli. Life (Basel, Switzerland), 12(12), 2077.

Kosmidis, C., Schindler, B. D., Jacinto, P. L., Patel, D., Bains, K., Seo, S. M., & Kaatz, G. W. (2012). Expression of multidrug resistance efflux pump genes in clinical and environmental isolates of Staphylococcus aureus. International journal of antimicrobial agents, 40(3), 204–209.

Kourtesi, C., Ball, A. R., Huang, Y. Y., Jachak, S. M., Vera, D. M., Khondkar, P., Gibbons, S., Hamblin, M. R., & Tegos, G. P. (2013). Microbial efflux systems and inhibitors: approaches to drug discovery and the challenge of clinical implementation. The open microbiology journal, 7, 34–52.

Kourtis, A. P., Hatfield, K., Baggs, J., Mu, Y., See, I., Epson, E., Nadle, J., Kainer, M. A., Dumyati, G., Petit, S., Ray, S. M., Emerging Infections Program MRSA author group, Ham, D., Capers, C., Ewing, H., Coffin, N., McDonald, L. C., Jernigan, J., & Cardo, D. (2019). Vital Signs: Epidemiology and Recent Trends in Methicillin-Resistant and in Methicillin-Susceptible Staphylococcus aureus Bloodstream Infections - United States. MMWR. Morbidity and mortality weekly report, 68(9), 214–219.

Kraemer, S. A., Ramachandran, A., & Perron, G. G. (2019). Antibiotic Pollution in the Environment: From Microbial Ecology to Public Policy. Microorganisms, 7(6), 180.

Kraemer, S. A., Ramachandran, A., & Perron, G. G. (2019). Antibiotic Pollution in the Environment: From Microbial Ecology to Public Policy. Microorganisms, 7(6), 180.

Kumar, K., Gupta, S.C., Chander, Y., & Singh, A.K. (2005). Antibiotic Use in Agriculture and Its Impact on the Terrestrial Environment. Advances in Agronomy, 87, 1-54.

Landecker, H. (2019). Antimicrobials before antibiotics: war, peace, and disinfectants. Palgrave Commun 5, 45.

Lee, H. Y., Chen, C. L., Wang, S. B., Su, L. H., Chen, S. H., Liu, S. Y., Wu, T. L., Lin, T. Y., & Chiu, C. H. (2011). Imipenem heteroresistance induced by imipenem in multidrug-resistant Acinetobacter baumannii: mechanism and clinical implications. International journal of antimicrobial agents, 37(4), 302–308.

Lerminiaux, N. A., & Cameron, A. D. S. (2019). Horizontal transfer of antibiotic resistance genes in clinical environments. Canadian journal of microbiology, 65(1), 34–44.

Levin, B. R., & Rozen, D. E. (2006). Non-inherited antibiotic resistance. Nature reviews. Microbiology, 4(7), 556–562.

Ligon B. L. (2004). Sir Howard Walter Florey--the force behind the development of penicillin. Seminars in pediatric infectious diseases, 15(2), 109–114. Wen, R., Li, C., Zhao, M., Wang, H., & Tang, Y. (2022). Withdrawal of antibiotic growth promoters in China and its impact on the foodborne pathogen Campylobacter coli of swine origin. Frontiers in microbiology, 13, 1004725.

Liu, H., Meng, L., Dong, L., Zhang, Y., Wang, J., & Zheng, N. (2021). Prevalence, Antimicrobial Susceptibility, and Molecular Characterization of Escherichia coli Isolated From Raw Milk in Dairy Herds in Northern China. Frontiers in microbiology, 12, 730656.

Liu, J., Zhu, Y., Jay-Russell, M., Lemay, D. G., & Mills, D. A. (2020). Reservoirs of antimicrobial resistance genes in retail raw milk. Microbiome, 8(1), 99.

Lo-Ten-Foe, J. R., de Smet, A. M., Diederen, B. M., Kluytmans, J. A., & van Keulen, P. H. (2007). Comparative evaluation of the VITEK 2, disk diffusion, etest, broth microdilution, and agar dilution susceptibility testing methods for colistin in clinical isolates, including heteroresistant Enterobacter cloacae and Acinetobacter baumannii strains. Antimicrobial agents and chemotherapy, 51(10), 3726–3730.

Machado, D., Antunes, J., Simões, A., Perdigão, J., Couto, I., McCusker, M., Martins, M., Portugal, I., Pacheco, T., Batista, J., Toscano, C., & Viveiros, M. (2018). Contribution of efflux to colistin heteroresistance in a multidrug resistant Acinetobacter baumannii clinical isolate. Journal of medical microbiology, 67(6), 740–749.

Marshall, B. M., & Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. Clinical microbiology reviews, 24(4), 718–733.

Martínez J. L. (2017). Effect of antibiotics on bacterial populations: a multi-hierachical selection process. F1000Research, 6, 51.

Mbindyo, C. M., Gitao, G. C., Plummer, P. J., Kulohoma, B. W., Mulei, C. M., & Bett, R. (2021). Antimicrobial Resistance Profiles and Genes of Staphylococci Isolated from Mastitic Cow's Milk in Kenya. Antibiotics (Basel, Switzerland), 10(7), 772.

McEwen, S. A., & Collignon, P. J. (2018). Antimicrobial Resistance: a One Health Perspective. Microbiology spectrum, 6(2), 10.1128/microbiolspec.ARBA-0009-2017.

McPhee, J.B., Tamber, S., Brazas, M.D., Lewenza, S., Hancock, R.E.W. (2009) Antibiotic Resistance Due to Reduced Uptake. In Antimicrobial Drug Resistance: Mechanisms of Drug Resistance; Mayers, D.L., Ed.; Humana Press: Totowa, NJ, USA, pp. 97–110.

Meek, R. W., Vyas, H., & Piddock, L. J. (2015). Nonmedical Uses of Antibiotics: Time to Restrict Their Use? PLoS biology, 13(10), e1002266.

Miyachiro, M. M., Contreras-Martel, C., & Dessen, A. (2019). Penicillin-Binding Proteins (PBPs) and Bacterial Cell Wall Elongation Complexes. Sub-cellular biochemistry, 93, 273–289.

Mulcahy, L. R., Burns, J. L., Lory, S., & Lewis, K. (2010). Emergence of Pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. Journal of bacteriology, 192(23), 6191–6199.

Nadeem, S. F., Gohar, U. F., Tahir, S. F., Mukhtar, H., Pornpukdeewattana, S., Nukthamna, P., Moula Ali, A. M., Bavisetty, S. C. B., & Massa, S. (2020). Antimicrobial resistance: more than 70 years of war between humans and bacteria. Critical reviews in microbiology, 46(5), 578–599.

Naqvi, S.A., Nadeem, S., Komal, S., Naqvi, S.A., Mubarik, M.S., Qureshi, S.Y., Ahmad, S., Abbas, A.H., Zahid, M., Khan, N.H., Raza, S.S., & Aslam, N. (2019). Antioxidants: Natural Antibiotics. Antioxidants.

Neela, F.A., Nonaka, L., Rahman, M.H. & Suzuki S. (2009). Transfer of the chromosomally encoded tetracycline resistance gene tet(M) from marine bacteria to Escherichia coli and Enterococcus faecalis . World J Microbiol Biotechnol 25, 1095–1101.

Nhung, N. T., Cuong, N. V., Thwaites, G., & Carrique-Mas, J. (2016). Antimicrobial Usage and Antimicrobial Resistance in Animal Production in Southeast Asia: A Review. Antibiotics (Basel, Switzerland), 5(4), 37.

Nweze, E. I., & Eze, E. E. (2009). Justification for the use of Ocimum gratissimum L in herbal medicine and its interaction with disc antibiotics. BMC complementary and alternative medicine, 9, 37.

Olivares, J., Bernardini, A., Garcia-Leon, G., Corona, F., B Sanchez, M., & Martinez, J. L. (2013). The intrinsic resistome of bacterial pathogens. Frontiers in microbiology, 4, 103.

Owusu-Kwarteng, J., Wuni, A., Akabanda, F., Tano-Debrah, K., & Jespersen, L. (2017). Prevalence, virulence factor genes and antibiotic resistance of Bacillus cereus sensu lato isolated from dairy farms and traditional dairy products. BMC microbiology, 17(1), 65.

Palma, E., Tilocca, B., & Roncada, P. (2020). Antimicrobial Resistance in Veterinary Medicine: An Overview. International journal of molecular sciences, 21(6), 1914.

Paquola, A. C. M., Asif, H., Pereira, C. A. B., Feltes, B. C., Bonatto, D., Lima, W. C., & Menck, C. F. M. (2018). Horizontal Gene Transfer Building Prokaryote Genomes: Genes Related to Exchange Between Cell and Environment are Frequently Transferred. Journal of molecular evolution, 86(3-4), 190–203.

Park, I. J., Lee, W. G., Shin, J. H., Lee, K. W., & Woo, G. J. (2008). VanB phenotype-vanA genotype Enterococcus faecium with heterogeneous expression of teicoplanin resistance. Journal of clinical microbiology, 46(9), 3091–3093.

Partridge S. R. (2015). Resistance mechanisms in Enterobacteriaceae. Pathology, 47(3), 276–284.

Parvin, M. S., Talukder, S., Ali, M. Y., Chowdhury, E. H., Rahman, M. T., & Islam, M. T. (2020). Antimicrobial Resistance Pattern of Escherichia coli Isolated from Frozen Chicken Meat in Bangladesh. Pathogens (Basel, Switzerland), 9(6), 420.

Pedonese, F., Verani, G., Torracca, B., Turchi, B., Felicioli, A., & Nuvoloni, R. (2019). Effect of an Italian propolis on the growth of Listeria monocytogenes, Staphylococcus aureus and Bacillus cereus in milk and whey cheese. Italian journal of food safety, 8(4), 8036.

Peláez, T., Cercenado, E., Alcalá, L., Marín, M., Martín-López, A., Martínez-Alarcón, J., Catalán, P., Sánchez-Somolinos, M., & Bouza, E. (2008). Metronidazole resistance in Clostridium difficile is heterogeneous. Journal of clinical microbiology, 46(9), 3028–3032.

Píriz Durán, S., Kayser, F. H., & Berger-Bächi, B. (1996). Impact of sar and agr on methicillin resistance in Staphylococcus aureus. FEMS microbiology letters, 141(2-3), 255–260.

Poirel, L., Madec, J. Y., Lupo, A., Schink, A. K., Kieffer, N., Nordmann, P., & Schwarz, S. (2018). Antimicrobial Resistance in Escherichia coli. Microbiology spectrum, 6(4), 10.1128/microbiolspec.ARBA-0026-2017.

Prabhakar, S. V. R. K., Sano, D., and Srivastava, N. (2010). “Food safety in the Asia-Pacific Region: current status, policy perspectives and a way forward,” in Sustainable Consumption and Production in the Asia-Pacific Region: Effective Responses in a Resource Constrained World White Paper III (Hayama: Institute for Global Environmental Strategies), 215–298.

Public Health Agency of Canada. (2017). Tackling Antimicrobial Resistance and Antimicrobial Use: A Pan-Canadian Framework for Action; Public Health Agency of Canada: Ottawa, ON, Canad

Pumbwe, L., & Piddock, L. J. (2000). Two efflux systems expressed simultaneously in multidrug-resistant Pseudomonas aeruginosa. Antimicrobial agents and chemotherapy, 44(10), 2861–2864.

Quaik, S., Embrandiri, A., Ravindran, B., Hossain, K., Al-Dhabi, N.A., Arasu, M.V., Ignacimuthu, S., & Ismail, N. (2020). Veterinary antibiotics in animal manure and manure laden soil: Scenario and challenges in Asian countries. Journal of King Saud University - Science, 32, 1300-1305.

Ramirez, M. S., & Tolmasky, M. E. (2010). Aminoglycoside modifying enzymes. Drug resistance updates: reviews and commentaries in antimicrobial and anticancer chemotherapy, 13(6), 151–171.

Rigourd, V., Barnier, J. P., Ferroni, A., Nicloux, M., Hachem, T., Magny, J. F., Lapillonne, A., Frange, P., Nassif, X., & Bille, E. (2018). Recent actuality about Bacillus cereus and human milk bank: a new sensitive method for microbiological analysis of pasteurized milk. European journal of clinical microbiology & infectious diseases: official publication of the European Society of Clinical Microbiology, 37(7), 1297–1303.

Rossi, G. A. M., Silva, H. O., Aguilar, C. E. G., Rochetti, A. L., Pascoe, B., Méric, G., Mourkas, E., Hitchings, M. D., Mathias, L. A., de Azevedo Ruiz, V. L., Fukumasu, H., Sheppard, S. K., & Vidal, A. M. C. (2018). Comparative genomic survey of Bacillus cereus sensu stricto isolates from the dairy production chain in Brazil. FEMS microbiology letters, 365(3), 10.1093/femsle/fnx283.

Ryu, S. H., Park, S. G., Choi, S. M., Hwang, Y. O., Ham, H. J., Kim, S. U., Lee, Y. K., Kim, M. S., Park, G. Y., Kim, K. S., & Chae, Y. Z. (2012). Antimicrobial resistance and resistance genes in Escherichia coli strains isolated from commercial fish and seafood. International journal of food microbiology, 152(1-2), 14–18.

Santos, L., & Ramos, F. (2018). Antimicrobial resistance in aquaculture: Current knowledge and alternatives to tackle the problem. International journal of antimicrobial agents, 52(2), 135–143.

Sarmah, A. K., Meyer, M. T., & Boxall, A. B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere, 65(5), 725–759.

Sasidharan, S., Prema, B., & Yoga, L. L. (2011). Antimicrobial drug resistance of Staphylococcus aureus in dairy products. Asian Pacific journal of tropical biomedicine, 1(2), 130–132.

Sawa, T., Kooguchi, K., & Moriyama, K. (2020). Molecular diversity of extended-spectrum β-lactamases and carbapenemases, and antimicrobial resistance. Journal of intensive care, 8, 13.

Sharun, K., Dhama, K., Tiwari, R., Gugjoo, M.B., Iqbal Yatoo, M., Patel, S.K., Pathak, M., Karthik, K., Khurana, S.K., Singh, R., Puvvala, B., Amarpal, Singh, R., Singh, K.P, Chaicumpa, W. Advances in therapeutic and managemental approaches of bovine mastitis: a comprehensive review. Vet Q. 2021 Dec;41(1):107-136.

Silva, J. G. da., Camargo, A. C., Melo, R. P. B. de., Aragão, B. B., Oliveira, J. M. B. de., Sena, M. J. de., Nero, L. A., Mota, R. A. (2022) A positive staphylococcus spp. in bovine mastitis, milkers, milking environment, and the circulation of diferent MRSA clones at dairy cows farms in the northeast region of Brazil," Ciˆencia Rural, 52(3): e20210008, 2022. tab, ilus .

Singh, V., Bala, M., Kakran, M., & Ramesh, V. (2012). Comparative assessment of CDS, CLSI disc diffusion and Etest techniques for antimicrobial susceptibility testing of Neisseria gonorrhoeae: a 6-year study. BMJ open, 2(4), e000969.

Sköld, O. (2011). Aminoglycosides. In Antibiotics and Antibiotic Resistance; John Wiley & Sons: Hoboken, NJ, USA, Volume 6,pp. 103–113.

So, A. D., Shah, T. A., Roach, S., Ling Chee, Y., & Nachman, K. E. (2015). An Integrated Systems Approach is Needed to Ensure the Sustainability of Antibiotic Effectiveness for Both Humans and Animals. The Journal of law, medicine & ethics: a journal of the American Society of Law, Medicine & Ethics, 43 Suppl 3, 38–45.

Souza, R. F. S., Rault, L., Seyffert, N., Azevedo, V., Le Loir, Y., & Even, S. (2018). Lactobacillus casei BL23 modulates the innate immune response in Staphylococcus aureus-stimulated bovine mammary epithelial cells. Beneficial microbes, 9(6), 985–995.

Superti, S. V., Martins, D.deS., Caierão, J., Soares, F.daS., Prochnow, T., & Zavascki, A. P. (2009). Indications of carbapenem resistance evolution through heteroresistance as an intermediate stage in Acinetobacter baumannii after carbapenem administration. Revista do Instituto de Medicina Tropical de Sao Paulo, 51(2), 111–113.

Tanaka, Y., Hipolito, C. J., Maturana, A. D., Ito, K., Kuroda, T., Higuchi, T., Katoh, T., Kato, H. E., Hattori, M., Kumazaki, K., Tsukazaki, T., Ishitani, R., Suga, H., & Nureki, O. (2013). Structural basis for the drug extrusion mechanism by a MATE multidrug transporter. Nature, 496(7444), 247–251.

Tang, Q., Song, P., Li, J., Kong, F., Sun, L., & Xu, L. (2016). Control of antibiotic resistance in China must not be delayed: The current state of resistance and policy suggestions for the government, medical facilities, and patients. Bioscience trends, 10(1), 1–6.

Tang, S. S., Apisarnthanarak, A., & Hsu, L. Y. (2014). Mechanisms of β-lactam antimicrobial resistance and epidemiology of major community- and healthcare-associated multidrug-resistant bacteria. Advanced drug delivery reviews, 78, 3–13.

Tato, M., Morosini, M., García, L., Albertí, S., Coque, M. T., & Cantón, R. (2010). Carbapenem Heteroresistance in VIM-1-producing Klebsiella pneumoniae isolates belonging to the same clone: consequences for routine susceptibility testing. Journal of clinical microbiology, 48(11), 4089–4093.

Van Boeckel, T. P., Brower, C., Gilbert, M., Grenfell, B. T., Levin, S. A., Robinson, T. P., Teillant, A., & Laxminarayan, R. (2015). Global trends in antimicrobial use in food animals. Proceedings of the National Academy of Sciences of the United States of America, 112(18), 5649–5654.

van Hal, S. J., Wehrhahn, M. C., Barbagiannakos, T., Mercer, J., Chen, D., Paterson, D. L., & Gosbell, I. B. (2011). Performance of various testing methodologies for detection of heteroresistant vancomycin-intermediate Staphylococcus aureus in bloodstream isolates. Journal of clinical microbiology, 49(4), 1489–1494.

Van Puyvelde, S., Deborggraeve, S., & Jacobs, J. (2018). Why the antibiotic resistance crisis requires a One Health approach. The Lancet. Infectious diseases, 18(2), 132–134.

Vasala, A., Hytönen, V. P., & Laitinen, O. H. (2020). Modern Tools for Rapid Diagnostics of Antimicrobial Resistance. Frontiers in cellular and infection microbiology, 10, 308.

Vijayakumar, R. & Kannan, Venkatesa & C. Manoharan, (2011). Minimum Inhibitory Concentration Determination of Chlorhexidine gluconate against Pharmaceutical Clean Room Fungal Isolates. Asian Journal of Pharmaceutical and Health Sciences. 4. 167 -170.

Wallinga, D., Smit, L. A. M., Davis, M. F., Casey, J. A., & Nachman, K. E. (2022). A Review of the Effectiveness of Current US Policies on Antimicrobial Use in Meat and Poultry Production. Current environmental health reports, 9(2), 339–354.

Williams-Nguyen, J., Sallach, J. B., Bartelt-Hunt, S., Boxall, A. B., Durso, L. M., McLain, J. E., Singer, R. S., Snow, D. D., & Zilles, J. L. (2016). Antibiotics and Antibiotic Resistance in Agroecosystems: State of the Science. Journal of environmental quality, 45(2), 394–406.

Wright G. D. (2010). The antibiotic resistome. Expert opinion on drug discovery, 5(8), 779–788.

Wu, S., de Lencastre, H., & Tomasz, A. (1996). Sigma-B, a putative operon encoding alternate sigma factor of Staphylococcus aureus RNA polymerase: molecular cloning and DNA sequencing. Journal of bacteriology, 178(20), 6036–6042.

Xiao, K. Q., Li, B., Ma, L., Bao, P., Zhou, X., Zhang, T., & Zhu, Y. G. (2016). Metagenomic profiles of antibiotic resistance genes in paddy soils from South China. FEMS microbiology ecology, 92(3), fiw023.

Yamakawa, J., Aminaka, M., Okuzumi, K., Kobayashi, H., Katayama, Y., Kondo, S., Nakamura, A., Oguri, T., Hori, S., Cui, L., Ito, T., Jin, J., Kurosawa, H., Kaneko, K., & Hiramatsu, K. (2012). Heterogeneously vancomycin-intermediate Staphylococcus aureus (hVISA) emerged before the clinical introduction of vancomycin in Japan: a retrospective study. Journal of infection and chemotherapy: official journal of the Japan Society of Chemotherapy, 18(3), 406–409.

Yang, L., Shen, Y., Jiang, J., Wang, X., Shao, D., Lam, M. M. C., Holt, K. E., Shao, B., Wu, C., Shen, J., Walsh, T. R., Schwarz, S., Wang, Y., & Shen, Z. (2022). Distinct increase in antimicrobial resistance genes among Escherichia coli during 50 years of antimicrobial use in livestock production in China. Nature food, 3(3), 197–205.

Yu, J. S. L., Correia-Melo, C., Zorrilla, F., Herrera-Dominguez, L., Wu, M. Y., Hartl, J., Campbell, K., Blasche, S., Kreidl, M., Egger, A. S., Messner, C. B., Demichev, V., Freiwald, A., Mülleder, M., Howell, M., Berman, J., Patil, K. R., Alam, M. T., & Ralser, M. (2022). Microbial communities form rich extracellular metabolomes that foster metabolic interactions and promote drug tolerance. Nature microbiology, 7(4), 542–555.

Yusuf, U., Kotwal, S. K., Gupta, S., & Ahmed, T. (2018). Identification and antibiogram pattern of Bacillus cereus from the milk and milk products in and around Jammu region. Veterinary world, 11(2), 186–191.

Zalewska, M., Błażejewska, A., Czapko, A., & Popowska, M. (2021). Antibiotics and Antibiotic Resistance Genes in Animal Manure - Consequences of Its Application in Agriculture. Frontiers in microbiology, 12, 610656.

Zampieri M. (2021). The genetic underground of antibiotic resistance. Science (New York, N.Y.), 371(6531), 783–784.

Zhang, F., & Cheng, W. (2022). The Mechanism of Bacterial Resistance and Potential Bacteriostatic Strategies. Antibiotics (Basel, Switzerland), 11(9), 1215.

Zhao, S., Chen, J., Fei, P., Feng, H., Wang, Y., Ali, M. A., Li, S., Jing, H., & Yang, W. (2020). Prevalence, molecular characterization, and antibiotic susceptibility of Bacillus cereus isolated from dairy products in China. Journal of dairy science, 103(5), 3994–4001.

Zhumakayeva, A., Zhubatkanova, A., Asauova, Z., Tokayeva, M., & Kemeshov, Z. (2023). Efficiency of probiotic culture consortium application for disinfection of dairy farm premises and prevention of mastitis in cows. Journal of advanced veterinary and animal research, 10(2), 185–195.

Chapter 2

Objectives and thesis outline

Chapter 2. Objectives and outline of the thesis

2.1 Objective

This thesis aimed to answer the following three questions: (1) To investigate the alterations of milk microbiota and the resistance genes following treatment with cephalosporins, and to evaluate the influences of antibiotics to maintain the rational use of antibiotics. (2) To investigate the effects of cephalosporins on the feces microbiota and antibiotic resistance genes of dairy cows with mastitis. (3) To screen for heteroresistance and explore the reason of ceftiofur heteroresistance in *Staphylococcus spp*. isolates obtained from raw milk.

2.2 Outline of the thesis

Chapter 1: General introduction

Chapter 2: Objectives and outline of thesis

Chapter 3 (Article 1): Effect of therapeutic administration of β-lactam antibiotics on the bacterial community and antibiotic resistance patterns in milk of dairy cows with clinical mastitis.

Dong L, Meng L, Liu H, Wu H, Hu H, Zheng N, Wang J, Schroyen M. Effect of therapeutic administration of β-lactam antibiotics on the bacterial community and antibiotic resistance patterns in milk. J Dairy Sci. 2021 Jun;104(6):7018-7025.

Chapter 4 (Article 2): Effect of cephalosporin treatment on the microbiota and antibiotic resistance genes in feces of dairy cows with clinical mastitis.

Dong L, Meng L, Liu H, Wu H, Schroyen M, Zheng N, Wang J. Effect of Cephalosporin Treatment on the Microbiota and Antibiotic Resistance Genes in Feces of Dairy Cows with Clinical Mastitis. Antibiotics (Basel). 2022 Jan 17;11(1):117.

Chapter 5 (Article 3):

The study on the heteroresistance to cephalosporins of Staphylococcus in milk from mastitis suffering cows. (To be submitted)

Chapter 6: General discussion, conclusion and perspectives

Chapter 3

Effect of therapeutic administration of β-lactams antibiotics on the bacterial community and antibiotic resistance patterns in milk of dairy cows with mastitis cows

Chapter 3. Effect of therapeutic administration of β-lactams antibiotics on the bacterial community and antibiotic resistance patterns in milk of dairy cows with mastitis

Lei Dong,[1,2,3*] Lu Meng,[1,2*] Huimin Liu,[1,2] Haoming Wu,[1,2] Haiyan Hu,[1,2] Nan Zheng,[1,2] Jiaqi Wang,[1,2†] and Martine Schroyen[3]

[1]Key Laboratory of Quality and Safety Control for Milk and Dairy Products of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[2]Laboratory of Quality and Safety Risk Assessment for Dairy Products of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[3]Precision Livestock and Nutrition Laboratory, Teaching and Research Centre (TERRA), Gembloux Agro-Bio Tech, University of Liège, Gembloux 5030, Belgium

[*] Authors who contribute equally to this work.

[*]Correspondence: jiaqiwang@vip.163.com

Running title: Effect of cephalosporins on the milk microbiome

Adapted from the reference: Dong L, Meng L, Liu H, et al. Effect of therapeutic administration of β-lactam antibiotics on the bacterial community and antibiotic resistance patterns in milk. J Dairy Sci. 2021;104(6):7018-7025.

3.1 Abstract

The effect of β-lactams antibiotics given to dairy cows on the milk microbiome is still not clear. The object of this research was to investigate the effect of two commonly used cephalosporins on the milk microbiota and the antibiotic resistance genes in the milk. The milk samples were collected from 7 dairy cows at the period before medication (day 0), medication (days 1, 2, 3), withdrawal period (days 4, 6, 8) and the period after withdrawal (days 9, 11, 13, 15). The milk samples were applied to explore the microbiota changes using 16S rRNA sequencing, and antibiotic resistance patterns were investigated by qPCR. The microbiota richness and diversity in each sample were calculated using the Chao 1 (richness), Shannon and Simpson (diversity) index. The cephalosporins treatment significantly lowered the Simpson diversity value at the period of withdrawal ($p < 0.05$). Members of the *Enterobacter* genera were the most affected bacteria associated with mastitis ($p < 0.05$). Meanwhile, antibiotic resistance genes in the milk were also influenced by antibiotic treatment. The cephalosporins treatment raised the relative proportion of *bla*TEM in milk samples at the period of withdrawal ($p < 0.05$). Therefore, the treatment of cephalosporins led to significant change in the milk microbiota and increase of β-lactam resistance gene in the milk at the time of withdrawal period.

Key words: antibiotic resistance gene; cephalosporin; dairy cow mastitis; milk microbiota

3.2 Introduction

Bovine mastitis, inflammation of the udder, is mainly caused by intramammary invasion of bacteria in the udder, which is one of the most prevalent diseases in dairy cows. Bovine mastitis can reduce milk production and milk quality, increase veterinary expenses, and diminish animal welfare (Halasa et al., 2007; Hogeveen et al., 2011; Heikkilä et al., 2012; Peters et al., 2015). Mastitis also accounts for the highest proportion of antibiotic use on dairy farms (Stevens et al., 2016). It is reported that 70% of antibiotics used to treat dairy cows are used to treat bovine mastitis in France. The average number of days each cow received antibiotic treatment for mastitis per year from 2005 to 2012 is 1.41 (Kuipers et al., 2016).

Nowadays, the abuse of antibiotics has become a major concern for public health, and the high number of antibiotic resistant bacteria has been found in different environments samples and even in foods (Flórez et al., 2017). Antibiotic resistance is considered as one of the biggest risks to public in the world. The prevalence of antibiotic resistant bacteria (ARB) in food has led to a concern that food may be a reservoir of antibiotic resistance genes (ARGs) and spread antibiotic resistance (Marshall et al., 2011). In recent years, resistance genes in ARB isolated from dairy products have been founded (Devirgiliis et al., 2010; Soares-Santos et al., 2015). Moreover, the resistance level of the microbial community will affect the dynamics of other microbial community after antibiotic therapy, which results in an even more increased antibiotic resistance (Holman et

al., 2019). There is also a worry that animals used antibiotics may spread ARB and ARGs into the surrounding environment (Alexander et al., 2008).

Cephalosporins are used to treat various bacterial infections in both human and animals. In 26 countries monitored by European Surveillance of Veterinary Antimicrobial Consumption (ESVAC), the use of 3rd-and 4th-generation cephalosporins increased in recent years (Scoppetta et al., 2016). The number of cephalosporins sold in the United States for food-producing animals was 26611 kg in 2011. And FDA revealed that 28000 kg cephalosporins were sold for livestock in 2013 (FDA, 2011; Kanwar et al., 2014). Ceftiofur is the only 3rd-generation cephalosporin labeled for veterinary use in the USA, and is the drug of choice in the majority of dairy intramammary treatment of mastitis operations (USDA, 2014). Cefquinome is a 4th-generation cephalosporin that was developed only for veterinary use. Cefquinome has been accepted for the treatment of acute mastitis, respiratory tract diseases, calf septicemia, metritis-mastitis-agalactia syndrome in sows, and respiratory diseases in pigs in Europe and elsewhere outside the United States (CVMP, 2003; Uney et al., 2011). It is reported that the use of ceftiofur (3rd-generation cephalosporin) leads to the occurrence of *E. coli* resistant to 1st and 2nd-generation cephalosporins (Sato et al., 2014). Therefore, the influence of cephalosporins therapy on microbiota and the antibiotic resistome needs to be evaluated.

To our knowledge, few reports have investigated the effect of therapeutic administration of β-lactams antibiotics on the microbiota and antibiotic resistance patterns in the milk via 16S rDNA sequencing. Therefore, the purposes of the research were to investigate the alterations of milk microbiota and the resistance genes following treatment with cephalosporins. It is important to evaluate the influences of antibiotics to maintain the rational use of antibiotics.

3.3 Materials and methods

3.3.1 Sample collection

Milk samples were received from a dairy farm in Tianjin city, China. The cows were not exposed to antibiotics prior to the study. The enumeration of somatic cells is a common method to identify mastitis. In this dairy farm, the dairy cow was judged to be mastitis by a veterinarian based on the obvious symptoms of redness of either udder, milk curdling, discoloration, and when the somatic cell count in milk was more than 500,000 cells/mL. A total of 7 mastitis affected dairy cows were selected. The cows used had an average body weight of 560-686kg, were 105-226 days in milk and gave between 34.26-39.12 kg of milk of milk per day. Milk from these cows was collected from the udder into a 400mL-sterile plastic bottle through the sterilized milk cup after diagnose on day 0. The cows were then injected with ceftiofur into the muscle at the level of 2mg/kg body weight and cefquinome into the udder at the level of 0.75ng/kg body weight per day for 3 days. All dairy cows were then sampled at days 1, 2, 3, 4, 6, 8, 9, 11, 13, and 15. All milk samples were directly put on ice and transported to the laboratory. The SCC in milk returned to the normal values on d 4, and the dairy cows stopped

receiving the antibiotics. The milk was tested for antibiotic residues on d 9. Day 0 was classified as the period before medication; d 1 to 3 was classified as the period of medication; d 4 to 8 was classified as the withdrawal period; d 9 to 15 was classified the period after withdrawal. The milk sample was tested to assure there was no antibiotic residue.

3.3.2 DNA extraction

Total DNA was extracted from 500 mL of each milk sample using the HiPure Soil DNA Kits (Magen, Guangzhou, China) according to manufacturer's instructions. The DNA samples' quality and concentration were measured using the Qubit 3.0 DNA detection kit (Life Technologies, Grand Island, NY). These DNA samples were stored at -80 ℃ for further genotypic quantification.

3.3.3 PCR amplification

The V3-V4 region of the 16S rDNA was amplified by PCR (94°C for 2 min, then 98°C for 10 s, 62°C for 30 s, and 68°C for 30 s for 30 cycles, and finally extended at 68°C for 5 min) using primers 341F: CCTACGGGNGGCWGCAG; 806R: GGACTACHVGGGTATCTAAT (Guo et al., 2017). Triplicate PCR reactions were performed with 50 μL of the mixture containing 5 μL of 10 × kodakaraensis (KOD) buffer, 5 μL of 2 mM deoxyribonucleoside triphosphate (Dntp), 3 μL of 25 mM MgSO4, 1.5 μL of each primer (10 μM), 1 μL of KOD polymerase, and 100 ng of template DNA. All PCR reagents were from TOYOBO.

3.3.4 Illumina Novaseq 6000 sequencing

The amplicons were extracted from a 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using the ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, USA). Purified amplicons were concentrated in equimolar and paired-end sequenced (PE250) on the Illumina platform according to the standard protocols.

3.3.5 Statistical Analysis

Raw data including adapters or low-quality reads would have effect on the following assembly and analysis. Therefore, in order to obtain high quality clean reads, FASTP (Chen et al., 2018) (version 0.18.0) was used to further filter the original reads according to the following rules. The UPARSE (Edgar et al., 2013) (version 9.2.64) pipeline was used to cluster the effective tags into operational taxonomic units (OTUs) with similarity ≥ 97 %. PCA (principal component analysis) was performed using the Vegan package in R (Oksanen et al., 2010) (version 2.5.3). The Vegan package in R (Oksanen) (version 2.5.3) was used to calculate Jaccard and bray-curtis distance matrix. The microbiota richness and diversity in each sample were calculated with QIIME (Caporaso et al., 2010) (version 1.9.1) using the Chao 1 (richness), Shannon and Simpson (diversity) index. Alpha index comparison among groups was calculated by Tukey's HSD

test and Kruskal-Wallis H test using the Vegan package in R ((Oksanen et al., 2010) (version 2.5.3).

3.3.6 Quantification of antibiotic resistance genes

The number of 16 antibiotic resistance genes was evaluated using qPCR as described previously (Holman et al., 2018). In brief, genes conferring resistance to beta-lactams (*cfx*A, *bla*ROB, *bla*1 and *bla*TEM), aminoglycosides (*str*A and *str*B), macrolides [erm(A) and erm(B)], sulfonamides (sul1 and sul2), tetracyclines [*tet*(B), *tet*(C), *tet*(Q) and *tet*(H)] and vancomycin (*van*C and *van*G) were evaluated. The primer sequences used were as previously released in Huang et al. (Huang et al., 2019). These genes were reflected as proportions of the 16S rRNA gene, which was also quantified by qPCR. The 16S rRNA gene was amplified using the 357-F: 5'-CCTACGGGAGGCAGCAG-3' and 518-R: 5'-ATTACCGCGGCTGCTGG-3' primers that were also used to generate the 16s rRNA gene libraries.

3.4 Results

3.4.1 Animal weight gain, and 16S rRNA sequencing overview

None of the dairy cows in this research received any other antibiotic treatments. The dairy cows were weighed before the medication and after the withdrawal period. The growth rate of the dairy cows was not influenced by the antibiotic medication ($P > 0.05$). The raw reads of sequences per sample ranged from 17,321 to 113,589. After cleaning, the tags of sequences per sample ranged from 16,640 to 112,958. The average effective ratio was 91.06%. The OTU numbers ranged from 223 to 1,462, and the average OTU number was 713.

3.4.2 α-diversity

Microbial diversity within α-diversity was measured by the richness (Chao 1) and by diversity indices (Shannon and Simpson). The average values of the Shannon diversity index and the average Chao 1 index for the microbiota were similar among the different periods, which suggested the total number of species did not significantly increase the richness of the microbiota ($P > 0.05$). However, the mean values of the Simpson index had significant differences among the different periods ($P > 0.05$), which indicated that the antibiotic treatment might have had an effect on the microbial community diversity (Figure 3-1).

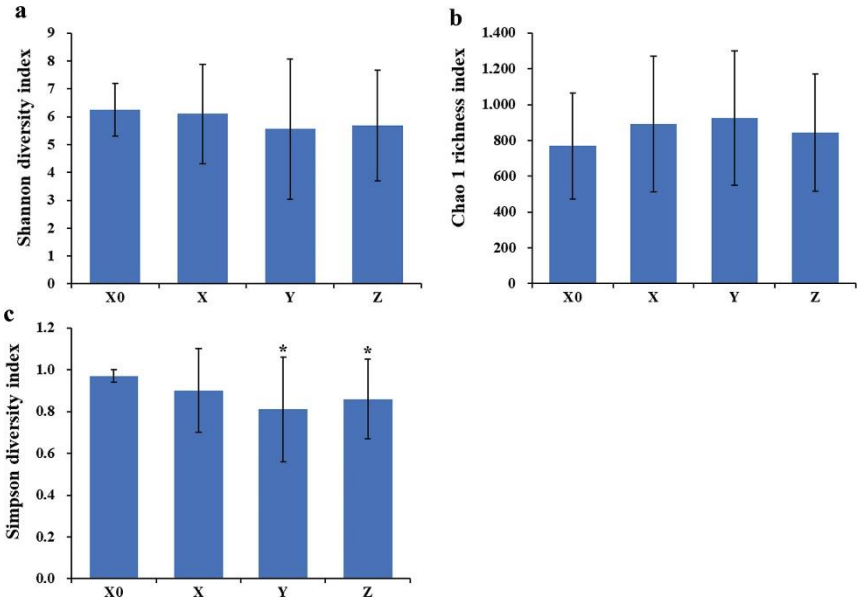


Figure 3-1. (a) Shannon diversity index of samples from 4 different periods. (b) Chao 1 richness index of samples from 4 different periods. (c) Simpson diversity index of samples from 4 different periods. *Indicates significant differences compared with X0 (P < 0.05). X0, X, Y, and Z indicate the periods of before medication, medication, withdrawal, and after withdrawal, respectively. Error bars indicate SD.

3.4.3 β-diversity

The β-diversity is the measurement of diversity between 2 or more groups. The higher the β-diversity, the greater the difference in species identity is among communities. The principal component analysis confirmed the correlation of the data: some samples from the 4 groups were separated by the first axis, which explained 50.58% of the species abundance variation (Figure 3-2). Based on the Bray-Curtis dissimilarities analysis, there were no significant differences in the period of before medication and medication ($P > 0.05$). The milk microbiota shifted significantly in the period of withdrawal ($P < 0.05$, Supplemental Table S1, https://figshare.com/articles/figure/supplement_table_xlsx/14167430). From the period before medication through the period after withdrawal time, there were no significant changes ($P > 0.05$) of the milk microbiota of the antibiotic-treated cows, indicating the recovery of the microbial community.

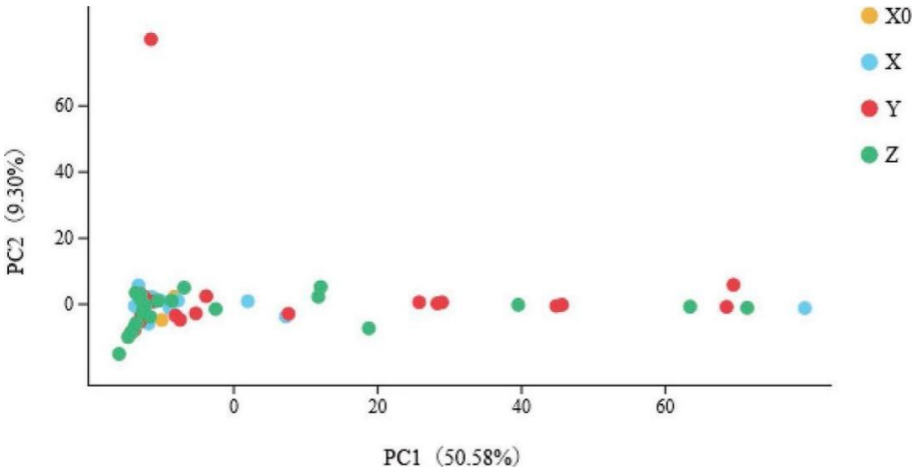


Figure 3-2. Principal coordinates (PC) analysis graph of the Bray dissimilarities in the milk samples between the sampling times. The percentages of change explained by the PC are displayed on the axes. X0, X, Y, and Z indicate the periods of before medication, medication, withdrawal, and after withdrawal, respectively.

3.4.4 Microbial community analysis

The milk samples collected from different periods were analyzed for the distribution of the microbial population at class-level and family-level distribution of the microbial population. The microbial community compositions of four periods were similar. Gammaproteobacteria was the most abundant bacterial class at the four periods, accounting for about 30% of the total species, followed by *Clostridia*, *Bacteroidia*, *Bacilli*, *Actinobacteria*, *Alphaproteobacteria*, *Oxyphotobacteria*, and *Verrucomicrobiae*. All these classes accounted for over 85% of all the bacteria (Figure 3-3). From the family level, *Preudomonadaceae*, *Burkholderiaceae*, *Ruminococcaceae*, and *Lachnospiraceae* were the four most abundant families, belonging to the *Clostridia* (Figure 3-4).

For some frequently detected common bacteria in milk, the use of antibiotics had effect on the bacterial composition of the milk microbiota. Most notably, the relative abundance of *Enterobacter* was significantly reduced at medication ($p < 0.05$) and decreased continuously from medication to the period of withdrawal (Figure 3-5a). The relative abundance of *Staphylococcus* and *Bacillus* did not differ significantly among different periods. Interestingly, the relative abundance of *Staphylococcus* and *Bacillus* was decreased from medication time to withdrawal time but both increased in the time after withdrawal (Figure 3-5b and Figure 3-5c). In comparison with the period of before medication, treatment with

antibiotics also significantly decreased the relative abundance of *Enterobacter*, *Blautia*, *Curvibacter*, *Bradyrhizobium*, *Enhydrobacter*, *and Gemmatimonas* at the genus level ($p < 0.05$, Figure 3-5d). The greatest dissimilarity among different periods occurred at the medication time (Figure 3-5d).

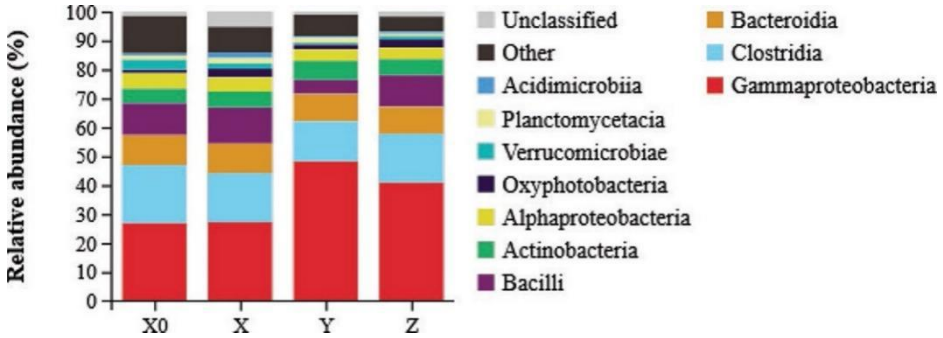


Figure 3-3. Milk microbiota composition at the class level of samples from different periods. The top 8 classes were described for each period, and all other classes were grouped as "Other" or "Unclassified." X0, X, Y, and Z indicate the periods of before medication, medication, withdrawal, and after withdrawal, respectively.

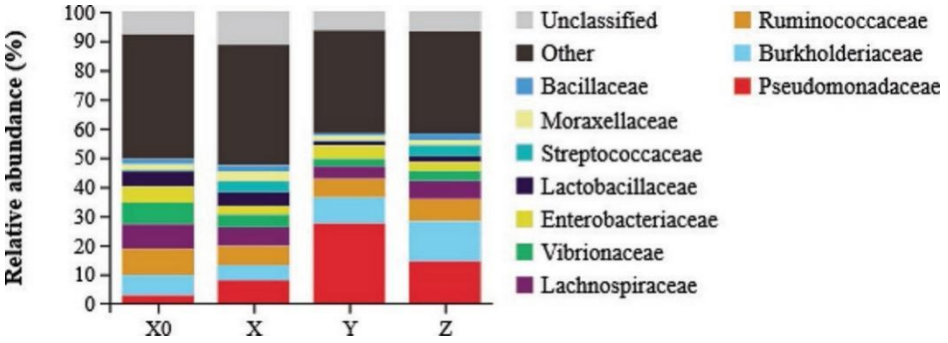


Figure 3-4. Milk microbiota composition at the family level of samples from different periods. The top 4 families were described for each period, and all other families were grouped as "Other" or "Unclassified." X0, X, Y, and Z indicate the periods of before medication, medication, withdrawal, and after withdrawal, respectively.

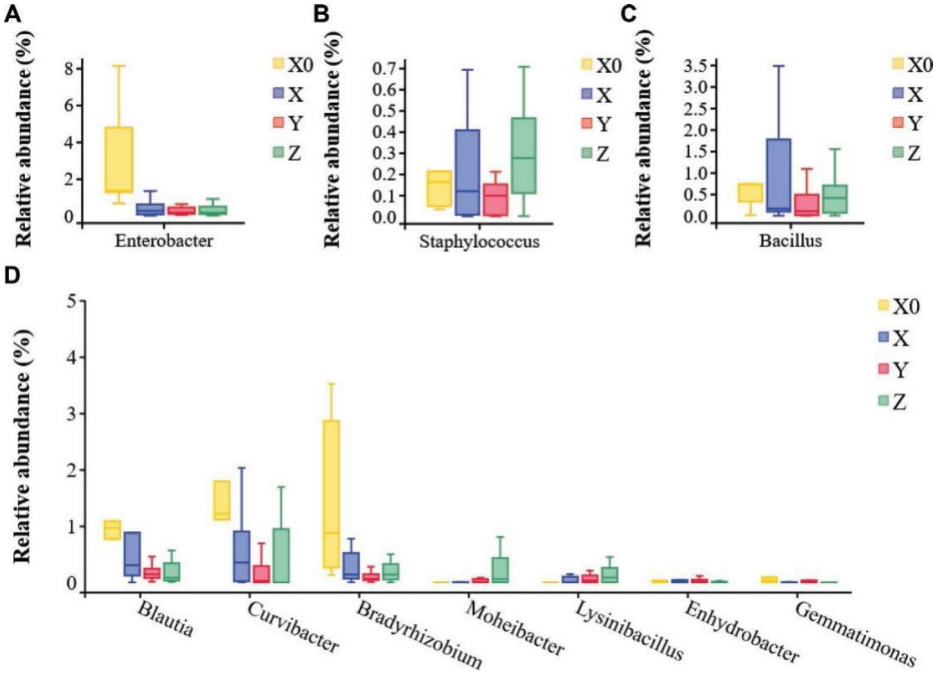


Figure 3-5. The relative abundance of the common bacterial genera in raw milk (a) Enterobacter, (b) Staphylococcus, (c) Bacillus, and (d) other bacterial genera with significantly different changes in milk at different periods (P < 0.05). X0, X, Y, and Z indicate the periods of before medication, medication, withdrawal, and after withdrawal, respectively. Error bars indicate SD.

3.4.5 Antibiotic resistance genes

The proportions of 16 ARG [*bla*1, *bla*ROB, *cfx*A, *bla*TEM, *str*A, *str*B, *erm* (A), *erm*(B), *tet*(C), *tet*(B), *tet*(Q), *tet*(H), *sul*1, *sul*2, *van*C, *van*G] in milk samples from 4 different sampling periods were quantified. Among these 16 resistance genes, only 7 genes [*cfx*A, *bla*TEM, *str*B, *tet*(A), *tet*(B), *tet*(C), *tet*(Q)] were higher than the detection limit in the milk samples (Figure 3-6). The ceftiofur and cefquinome significantly increased the proportion of *bla*TEM in the milk at withdrawal time (Figure 3-6). The relative abundance of *bla*TEM was significantly increased at the time after withdrawal compared with withdrawal time. Other resistance genes were not affected by antibiotic treatment (data not shown).

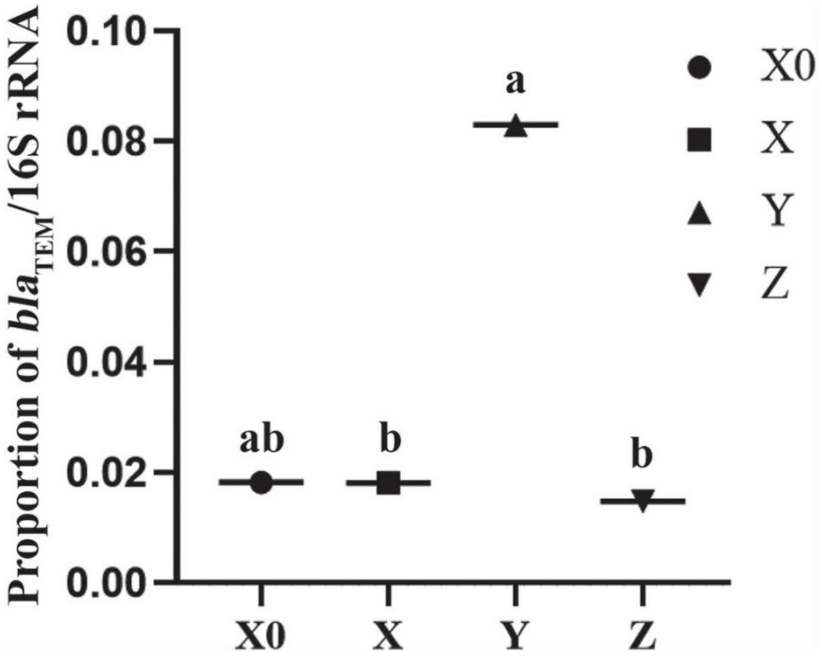


Figure 3-6. Proportion of the antibiotic resistance genes *bla*TEM compared to 16S rRNA gene. Different lowercase letters (a and b) suggest significantly different means (P < 0.05). X0, X, Y, and Z indicate the periods of before medication, medication, withdrawal, and after withdrawal, respectively.

3.5 Discussion

The analysis of the Bray-Curtis dissimilarities suggested that the milk microbiota structure shifted at withdrawal time ($p < 0.05$). Because this was the most immediate sampling time after administration, continuous antibiotic treatment may have had an impact on the cows. At the same time, it may be inferred that the use of antibiotic resulted in changes in the milk microbiota over three days rather than an immediate change (≤1d). The milk microbiota shifted significantly in the period of withdrawal ($p < 0.05$). This might be caused by the elevation in the phylum of *Proteobacteria* and the reduce in the phylum of *Firmicutes* and *Verrucomicrobia* (Additional file 1: Table S2, https://figshare.com/articles/figure/supplement_table_xlsx/14167430). It has been confirmed that some antimicrobials agents can reduce the diversity of gut microbiota of cows (Allen et al., 2011). However, the relative abundance of the microbiota increased at the period of withdrawal time, showing that the antibiotics suggested a prolonged antimicrobial effect (> 8 d).

The administration of cephalosporins can affect the bacterial composition of the

milk microbiota. It is reported that the *Bacteroides* strains isolated from various of human infections contained β-lactamase genes, which reduced sensitivity to abroad profiles of antibiotics (García et al., 2016). Especially, it was measured that the isolated *Bacteroides* strains were highly resistant to 1st and 2nd -generation cephalosporins and were moderately resistant when exposed to 3rd and 4th-generation cephalosporins (Nuria et al., 2008). In our study, the Enterobacter was significantly reduced at medication ($p < 0.05$) and decreased continuously from medication to the period of withdrawal (Figure 3-5a). This finding suggested the cephalosporins were effective in reducing the colonization of Enterobacter. However, a recent study has suggested appropriate veterinary use of a 3rd -generation cephalosporin can increase the occurrence of 1st and 2nd -generation cephalosporin-resistant *E. coli* in the rectal. The abuse of ceftiofur in veterinary therapy might elevate the risk of *E. coli* resistant to cephalosporin. Due to the flexibility of genome in *E. coli*, this organism has evolved into pathogenic strains capable of causing diseases, including bovine mastitis following fecal contamination of the teat skin. So, the cephalosporin should be used prudently and monitored carefully to prevent the spread of bacteria resistant to cephalosporin.

ARGs can be settled in mobile genetic elements (MGEs) and be transferred through horizontal gene transfer (HGT) from bacteria in food to human (Spanu et al., 2014). It is worth noting that this transfer can occur between the same or different species and may be associated with pathogenic bacteria and non-pathogenic bacteria (Spanu et al., 2010). In addition, HGT will be faster when the donor of the antibiotic resistance system and its recipient belong to the same family (Kruse et al., 1994). It is reported that the *tet*M genes commonly connected with MGEs were detected in 42% of *E. coli* isolated strains in raw milk in Poland and the risk of ARGs transferring from milk bacteria to human gastrointestinal coliform bacteria may be high (Godziszewska et al., 2018).

Previous research mainly focused on the presence of antibiotic resistance genes in feces, nasopharyngeal, rumen, and gut etc. rather than milk (Durso et al., 2017; Holman et al., 2019). However, as far as we know, this is the first evaluation of antibiotic resistance genes in the milk microbiome of mastitis affected dairy cow from before medication period to the period after withdrawal. Only *bla*TEM was significantly increased ($p < 0.05$) from medication time to withdrawal time. The *bla*TEM gene has been detected ubiquitously among *Enterobacteriaceae* (Lachmayr et al., 2009), *Stenotrophomonas maltophilia* (Matthew et al., 2000), *Neisseria gonorrhoeae* (Gianecini et al., 2015), *Haemophilus* parainfluenzae (García-Cobos et al., 2013), and *Pseudomonas aeruginosa* (Marchandin et al., 2000). Considering the proportion of *bla*TEM in milk during the withdrawal period in this study, it seems that the milk might be an important carrier of the *bla*TEM gene. The *bla*TEM gene has also been found in bulk tank milk (Sudarwanto et al., 2015). The cephalosporins changed the proportion of antibiotic resistance genes in the milk microbiome at different periods, which involved the other kinds of antibiotic resistance genes not only cephalosporins

resistance. However, the treatment of a kind of antibiotic can provide selective pressure to maintain other unassociated resistance genes by linking to MGEs (García et al., 2016).

3.6 Conclusion

This study provides a snapshot of the profile of the milk microbiota and ARGs affected by cephalosporins. The relative abundance of *Enterobacter* was significantly reduced along with the use of cephalosporins ($P < 0.05$). However, the relative abundance of the β-lactam gene *bla*TEM was increased at the period of withdrawal time. The effect of the cephalosporin treatment on the milk microbiota and resistome are worthy of further investigation.

3.7 Acknowledgements

This work was supported by the Scientific Research Project for Major Achievements of the Agricultural Science and Technology Innovation Program (Beijing, China; ASTIP no. CAASZDXT2019004), the Agricultural Science and Technology Innovation Program (Beijing, China; ASTIP-IAS12), Modern Agro-Industry Technology Research System of the PR China (Beijing, China; CARS-36), and Project of Risk Assessment on Raw Milk (Beijing, China; GJFP2019008). The authors declare no conflict of interest.

3.8 Supplemental information

Supplemental information is available in the online version of this article.

3.9 References

Alexander, T. W., L. J. Yanke, E. Topp, M. E. Olson, R. R. Read, D. W. Morck, and T. A. McAllister. (2008). Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant Escherichia coli bacteria in feedlot cattle. Appl. Environ. Microbiol. 74:4405–4416.

Allen, H. K., L. Torey, D. O. Bayles, H. Samuel, U. Y. Levine, A. David, and T. B. Stanton. (2011). Antibiotics in feed induce prophages in swine fecal microbiomes. Mbio. 2:1867–1877.

Avison, M. B., C. J. von Heldreich, C. S. Higgins, P. M. Bennett, and T. R. Walsh. (2000). A TEM-2 β-lactamase encoded on an active Tn1-like transposon in the genome of a clinical isolate of Stenotrophomonas maltophilia. J. Antimicrob. Chemother. 46:879–884.

Bradley, A. J., & Green, M. J. (2009). Factors affecting cure when treating bovine clinical mastitis with cephalosporin-based intramammary preparations. Journal of dairy science, 92(5), 1941–1953.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Peña, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335–336.

Chen, S., Y. Q. Zhou, Y. R. Chen, and J. Gu. (2018). Fastp: An ultrafast all-in-one fastq preprocessor. Bioinformatics 34: i884–i890.

CVMP. (2003). Cefquinome (extension to horses). Summary report (3). EMEA/MRL/ 883/03-final. European Agency for the Evaluation of Medicinal Products.

Denamur, E., Clermont, O., Bonacorsi, S., & Gordon, D. (2021). The population genetics of pathogenic Escherichia coli. Nature reviews. Microbiology, 19(1), 37–54.

Devirgiliis, C., S. Barile, A. Caravelli, D. Coppola, and G. Perozzi. (2010). Identification of tetracycline- and erythromycin-resistant Gram-positive cocci within the fermenting microflora of an Italian dairy food product. J. Appl. Microbiol. 109:313–323.

Durso, L. M., G. P. Harhay, J. L. Bono, and T. P. L. Smith. (2011). Virulence-associated and antibiotic resistance genes of microbial populations in cattle feces analyzed using a metagenomic approach. J. Microbiol. Methods 84:278–282.

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10:996–998.

FDA. (2011). Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. Department of Health and Human Services.

Flórez, A. B., V. Lucía, and M. Baltasar. (2017). A functional metagenomic analysis of tetracycline resistance in cheese bacteria. Front. Microbiol. 8:907.

García-Cobos, S., M. Arroyo, J. Campos, M. Pérez-Vázquez, B. Aracil, E. Cercenado, B. Orden, N. Lara, and J. Oteo. (2013). Novel mechanisms of resistance to β-lactam antibiotics in Hemophilus parainfluenzae: β-lactamase-negative ampicillin

resistance and inhibitor-resistant TEM β-lactamases. J. Antimicrob. Chemother. 68:1054–1059.

García, N., G. Gutiérrez, M. Lorenzo, J. E. García, S. Píriz, and A. Quesada. (2008). Genetic determinants for cfxA expression in Bacteroides strains isolated from human infections. J. Antimicrob. Chemother. 62:942–947.

Gianecini, R., C. Oviedo, A. Littvik, E. Mendez, and P. Galarza. (2015). Identification of TEM-135 β-lactamase in Neisseria gonorrhoeae strains carrying African and Toronto plasmids in Argentina. Antimicrob. Agents Chemother. 59:717–720.

Godziszewska, J., E. Pogorzelska-Nowicka, M. Brodowska, G. JaguraBurdzy, and A. Wierzbicka. (2018). Detection in raw cow's milk of coliform bacteria - reservoir of antibiotic resistance. Lebensm. Wiss. Technol. 93:634–640.

Guo, M., F. Wu, G. Hao, Q. Qi, R. Li, N. Li, L. Wei, and T. Chai. (2017). Bacillus subtilis improves immunity and disease resistance in rabbits. Front. Immunol. 8:354.

Halasa, T., K. Huijps, O. Østerås, and H. Hogeveen. (2007). Economic effects of bovine mastitis and mastitis management: A review. Vet. Q. 29:18–31.

Heikkilä, A.-M., J. I. Nousiainen, and S. Pyörälä. (2012). Costs of clinical mastitis with special reference to premature culling. J. Dairy Sci. 95:139–150.

Hogeveen, H., K. Huijps, and T. Lam. (2011). Economic aspects of mastitis: New developments. N. Z. Vet. J. 59:16–23.

Holman, D. B., E. Timsit, C. W. Booker, and T. W. Alexander. (2018). Injectable antimicrobials in commercial feedlot cattle and their effect on the nasopharyngeal microbiota and antimicrobial resistance. Vet. Microbiol. 214:140–147.

Holman, D. B., W. Yang, and T. W. Alexander. (2019). Antibiotic treatment in feedlot cattle: A longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. Microbiome 7:86.

Huang, X., J. Zheng, S. Tian, C. Liu, L. Liu, and L. Wei. (2019). Higher temperatures do not always achieve better antibiotic resistance gene removal in anaerobic digestion of swine manure. Appl. Environ. Microbiol. 85: e02878-18.

Johnson, T. A., R. D. Stedtfeld, Q. Wang, J. R. Cole, and J. M. Tiedje. (2016). Clusters of antibiotic resistance genes enriched together stay together in swine agriculture. MBio 7: e02214.

Kanwar, N., H. M. Scott, B. Norby, G. H. Loneragan, J. Vinasco, and J. L. Cottell. (2014). Impact of treatment strategies on cephalosporin and tetracycline resistance gene quantities in the bovine fecal metagenome. Sci. Rep. 29:5100.

Kruse, H., and H. Sørum. (1994). Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. 60:4015–4021.

Kuipers, A., W. J. Koops, and H. Wemmenhove. (2016). Antibiotic use in dairy herds in the Netherlands from 2005 to 2012. J. Dairy Sci. 99:1632–1648.

Lachmayr, K. L., L. J. Kerkhof, A. G. Dirienzo, C. M. Cavanaugh, and T. E. Ford. (2009). Quantifying nonspecific TEM β-lactamase (blaTEM) genes in a wastewater stream. Appl. Environ. Microbiol. 75:203–211.

Lang, I., M. Rose, E. Thomas, and E. Zschiesche. (2002). A field study of cefquinome for the treatment of pigs with respiratory disease. Rev. Med. Vet. (Toulouse) 153:575–580.

Marchandin, H., H. Jean-Pierre, C. De Champs, D. Sirot, H. Darbas, P. F. Perigault, and C. Carriere. (2000). Production of a tem-24 plasmid-mediated extended-spectrum β-lactamase by a clinical isolate of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 44:213–216.

Marshall, B. M., and S. B. Levy. (2011). Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24:718–733.

Oksanen, J., F. G. Blanchet, R. Roeland Kindt, P. Legendre, and H. Wagner. (2010). Vegan: Community ecology package. R package version 1.17-4. http://cran.r-project.org.

Peters, M. D. P., I. D. B. Silveira, and V. Fischer. (2015). Impact of subclinical and clinical mastitis on sensitivity to pain of dairy cows. Animal. 9:2024–2028.

Santamaría, J. (2011). Detection and diversity evaluation of tetracycline resistance genes in grassland-based production systems in Colombia, South America. Front. Microbiol. 2:252.

Sato, T., T. Okubo, M. Usui, S. I. Yokota, S. Izumiyama, and Y. Tamura. (2014). Association of veterinary third-generation cephalosporin use with the risk of emergence of extended-spectrum-cephalosporin resistance in Escherichia coli from dairy cattle in japan. PLoS One 9: e6101.

Scoppetta, F., T. Cenci, A. Valiani, R. Galarini, and M. Capuccella. (2016). Qualitative survey on antibiotic use for mastitis and antibiotic residues in Umbrian dairy herds. Large Anim. Rev. 22:11–18.

Soares-Santos, V., A. S. Barreto, and T. Semedo-Lemsaddek. (2015). Characterization of enterococci from food and food-related settings. J. Food Prot. 78:1320–1326.

Spanu, V., C. Scarano, F. Cossu, C. Pala, C. Spanu, and E. P. L. De Santis. (2014). Antibiotic resistance traits and molecular subtyping of Staphylococcus aureus isolated from raw sheep milk cheese: Staphylococcus aureus drug resistance. J. Food Sci. 79:M2066–M2071.

Spanu, V., S. Virdis, C. Scarano, F. Cossu, E. P. L. De Santis, and A. M. Cosseddu. (2010). Antibiotic resistance assessment in S. aureus strains isolated from raw sheep's milk cheese. Vet. Res. Commun. 34(Suppl. 1): S87–90.

Stevens, M., S. Piepers, and S. De Vliegher. (2016). Mastitis prevention and control practices and mastitis treatment strategies associated with the consumption of (critically important) antimicrobials on dairy herds in Flanders, Belgium. J. Dairy Sci. 99:2896–2903.

Sudarwanto, M., Ö. Akineden, S. Odenthal, M. Gross, and E. Usleber. (2015). Extended-spectrum β-lactamase (ESBL)–producing Klebsiella pneumoniae in bulk tank milk from dairy farms in indonesia. Foodborne Pathog. Dis. 12:585–590.

Uney, K., F. Altan, and M. Elmas. (2011). Development and validation of a high-performance liquid chromatography method for determination of cefquinome concentrations in sheep plasma and its application to pharmacokinetic studies. Antimicrob. Agents Chemother. 55:854–859.

USDA. (2014). Dairy 2014, Milk Quality, Milking Procedures, and Mastitis in the United States. USDA–APHIS–VS–CEAH–NAHMS

.

Chapter 4

Effect of Cephalosporin Treatment on the Microbiota and Antibiotic Resistance Genes in Feces of Dairy Cows with Clinical Mastitis

Chapter 4. Effect of Cephalosporin Treatment on the Microbiota and Antibiotic Resistance Genes in Feces of Dairy Cows with Clinical Mastitis

Lei Dong,[1,2,3*] Lu Meng,[1,2*] Huimin Liu,[1,2] Haoming Wu,[1,2] Nan Zheng,[1,2] Martine Schroyen[3] and Jiaqi Wang,[1,2†]

[1]Key Laboratory of Quality and Safety Control for Milk and Dairy Products of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[2]Laboratory of Quality and Safety Risk Assessment for Dairy Products of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[3]Precision Livestock and Nutrition Laboratory, Teaching and Research Centre (TERRA), Gembloux Agro-Bio Tech, University of Liège, Gembloux 5030, Belgium

[*] Authors who contribute equally to this work.

[*]Correspondence: jiaqiwang@vip.163.com

Running title: Effect of cephalosporins on the fecal microbiome

Adapted from the reference: Dong L, Meng L, Liu H, et al. Effect of Cephalosporin Treatment on the Microbiota and Antibiotic Resistance Genes in Feces of Dairy Cows with Clinical Mastitis. Antibiotics (Basel). 2022;11(1):117.

4.1 Abstract

Antibiotics are frequently used to treat dairy cows with mastitis. However, the potential effects of β-lactam antibiotics, such as cephalosporins, on the fecal microbiome of dairy cows with mastitis is largely unknown. The objective was to investigate the effects of ceftiofur and cefquinome on the fecal microbiota and antibiotic resistance genes of dairy cows with mastitis. The fecal samples were collected from 8 dairy cows at the following periods: the start day (Day 0), medication (Days 1, 2, and 3), withdrawal (Days 4, 6, 7, and 8), and recovery (Days 9, 11, 13, and 15). 16S rRNA gene sequencing was applied to explore the changes in microbiota, and qPCR was used to investigate the antibiotic resistance genes. The cephalosporin treatment significantly decreased the microbial diversity and richness, indicated by the decreased Shannon and Chao 1 indexes, respectively ($p < 0.05$). The relative abundance of *Bacteroides*, *Bacteroidaceae*, *Bacteroidales*, and *Bacteroidia* increased, and the relative abundance of *Clostridia*, *Clostridiales*, *Ethanoligenens*, and *Clostridium* IV decreased at the withdrawal period. The cephalosporin treatment increased the relative abundance of β-lactam resistance genes (*bla*TEM and *cfx*A) at the withdrawal period ($p < 0.05$). In conclusion, the cephalosporin treatment decreased the microbial diversity and richness at the medication period, and increased the relative abundance of two β-lactam resistance genes at the withdrawal period.

Keywords: dairy cow mastitis; cephalosporin; fecal microbiota; antibiotic resistance genes

4.2 Introduction

Mastitis is regarded as one of the most frequent diseases in the dairy cows. Mastitis can directly and/or indirectly affect milk hygienic quality (Halasa et al., 2007) and can lead to a substantial loss in milk production (Santos et al., 2004; Turk et al., 2012). The average economic losses due to mastitis are estimated at around USD 325 per cow per year (Huijps et al., 2008). About 24% of antibiotics in the dairy industry are used for mastitis treatment and around 44% for mastitis prevention (Kuipers et al., 2016). Antibiotic usage (both oral and injection) has a profound influence on the microbiome of animal feces, leading to an increase in the relative abundance of antibiotic resistance genes (Xiong et al., 2018; Holman et al., 2019). There is also a concern that due to the continued antibiotic use to prevent or treat mastitis, antimicrobial resistance will greatly increase the difficulty and cost of treatment (Kaniyamattam et al., 2020). It is reported that the chlortetracycline treatment promoted the abundance of tetracycline resistance genes such as *tet*(A) and *tet*(W) (Xiong et al., 2018). Antibiotic resistance genes (ARGs) can be transferred into the environment and pose a high risk to soil ecology and public health (Bengtsson-Palme et al., 2015). The abundance of

ARGs in fertilized greenhouse soils was higher than that in field soil (Fang et al., 2015).

Cephalosporins are frequently used in veterinary medicine to treat bacterial infections (Chambers et al., 2015). Ceftiofur is a third-generation cephalosporin that was approved for veterinary use to treat various Gram-negative bacterial infections. Ceftiofur is also one of the most used antibiotics in dairy cows to treat mastitis, metritis, and respiratory diseases (Foster et al., 2019), and is the only third-generation cephalosporin approved for veterinary use in the USA (USDA et al., 2014). Cefquinome is a fourth-generation cephalosporin developed solely for veterinary use. Cefquinome can treat the infections caused by *Staphylococcus aureus*, *Streptococcus suis Serotype* 2, and *Escherichia coli* (Wang et al., 2014; Zhou et al., 2015; Guo et al., 2016). It was reported that bacteria in animals can develop resistance to cefquinome (Li et al., 2016; Pehlivanoglu et al., 2016). A Swiss study found up to 44% of *Escherichia coli* isolates resistant to cefquinome (Stannarius et al., 2009). There is a reasonable concern that the use of cefquinome in cows could increase the expression of β-lactam genes, which may deliver resistance to cephalosporins (Chambers et al., 2015). Resistance to cephalosporin was related to the production of β-lactamases (Liu et al., 2007). It is reported that β-lactamase encoding genes, such as *bla*TEM, *bla*CMY, *bla*SHV, and *cfx*A, confer resistance to ceftiofur (Zhao et al., 2001; Chambers et al., 2015). To date, no research has investigated the effect of cephalosporins on the fecal microbiome of mastitis cows.

The objective was to investigate the alterations in fecal microbiota and the antibiotic resistance genes following cephalosporin treatment of mastitis cows.

4.3 Materials and methods

4.3.1 Animals and sample collections

Fecal samples were collected from a dairy farm in Tianjin city, China. The dairy cows were judged to suffer from clinical mastitis by a veterinarian based on the obvious symptoms of redness of either udder, milk curdling, discoloration, and when the somatic cell count in milk was more than 500,000 cells/mL. The somatic cell count was calculated by the California mastitis test (CMT). A total of 8 primiparous mastitis-affected Holstein dairy cows (one quarter was infected; 560–686 kg body weight; 105–226 days in milk; 34.26–39.12 kg of milk per day) were selected. The cows had not been treated with any antibiotics prior to the study. According to the uniform regulations of the dairy farm, these mastitis cows were housed individually in a well-ventilated barn and fed a totally mixed ration three times daily. Prior to antibiotic treatment, feces from these cows were collected from the rectum by the veterinarian, grabbed with sterile groves. Approximately 300 g of feces collected for the first time were discarded to prevent contamination. Then 100 g of the fresh samples were immediately placed into a 200 mL-sterile plastic bottle to avoid exposure to the environment. The

cows were then injected with ceftiofur sodium for injection (Qilu animal health products co., LTD, Shandong, China) into the muscle (2 mg/kg body weight), and with a cefquinome sulfate intermammary infusion (Qilu animal health products co., LTD, Shandong, China) into the teat canal of the mastitis-affected quarter (0.75 ng/kg body weight) by the veterinarian once per day from Day 1 to Day 3. The somatic cell counts in milk returned to normal values (<200 thousand cells/mL), and the dairy cows stopped receiving the antibiotics after Day 3. The milk was tested by Delvotest SP-NT (DSM Food Specialities R&D, Delft, The Netherlands) according the manufacturer's instructions to ensure that the antibiotics were not detected in the milk on Day 9. The feces of all dairy cows (n=8) were then sampled at Days 1, 2, 3, 4, 6, 8, 9, 11, 13, and 15 (Figure 4-1). Day 0 referred to the start day; Days 1 to 3 were classified as the medication period; Days 4 to 8 were classified as the withdrawal period; and Days 9 to 15 were classified as the recovery period.

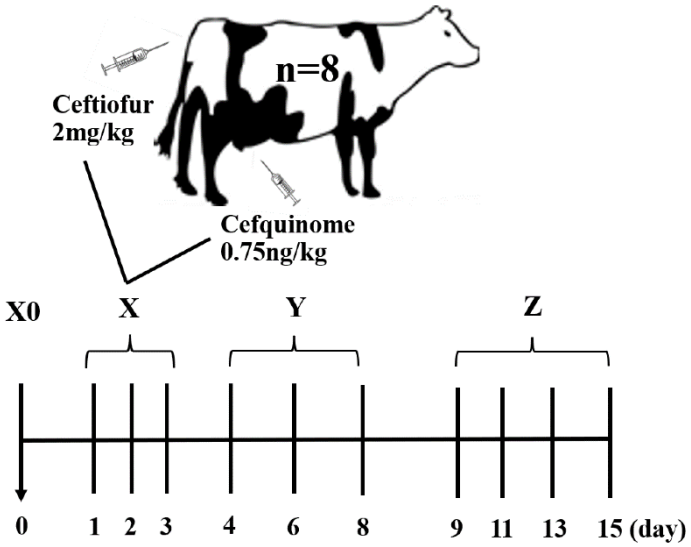


Figure 4-1. Timeline for fecal sampling. Sampling days are displayed above the black lines. The antibiotic treatments are noted at Days 1, 2, and 3. X0, X, Y, and Z indicate the periods Day 0, medication, withdrawal, and recovery, respectively.

4.3.2 DNA extraction

Total DNA was extracted from 500 mg of each fecal sample using the E.Z.N.A™ Mag-Bind Soil DNA Kit (OMEGA, Norcross, GA, USA), according to manufacturer's instructions. The DNA samples' quality and concentration were

measured using a Qubit 3.0 DNA detection kit (Life Technologies, Grand Island, NY, USA). These DNA samples were stored at −80 °C for further genotypic quantification.

4.3.3 PCR Amplification

The V3–V4 region of the 16S rDNA was amplified by PCR (94 °C for 3 min, 94 °C for 30 s, 45 °C for 20 s, and 65 °C for 30 s for 5 cycles). Illumina bridge PCR compatible primers were introduced in the second round of PCR amplification at 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s for 20 cycles, and finally extended at 72 °C for 5 min using primers 341F: CCTACGGGNGGCWGCAG; 805R: GACTACHVGGGTATCTAATCC (Su et al., 2015). Triplicate PCR reactions were performed with 30 μL of the mixture containing 15 μL of 2 × Hieff Robust PCR Master Mix, 1 μL of Primer F, 1 μL of primer R, 10–20 ng template DNA or PCR products, and H®2O was added to 30 μL. All PCR reagents were from TOYOBO, Japan.

4.3.4 Illumina Novaseq 6000 Sequencing

The amplicons were extracted from a 2% agarose gels and purified using the SanPrep DNA Gel Extraction Kit (SANGON Biotechnology, Shanghai, China), according to the manufacturer's instructions, and quantified using the ABI Step One Plus Real-Time PCR System (Life Technologies, Foster City, CA, USA). Purified amplicons were concentrated in equimolar and paired-end sequenced (PE250) on the Illumina platform according to the standard protocols.

4.3.5 Quantification of Antibiotic Resistance Genes

The quantity of 20 antibiotic resistance genes was evaluated using qPCR, as described previously (Holman et al., 2019). In brief, genes conferring resistance to beta-lactams (*cfx*A, *bla*ROB, *bla*CMY, *mec*A, *bla*CTX-M, *bla*1, and *bla*TEM), aminoglycosides (*str*A and *str*B), macrolides (*erm*(A) and *erm*(B)), sulfonamides (*sul*1 and *sul*2), tetracyclines (*tet*(A), *tet*(B), *tet*(C), *tet*(H) and *tet*(Q)), and vancomycin (*van*C and *van*G) were evaluated. The primer sequences used were as previously described in Huang et al. (Huang et al., 2019). These genes were normalized against the 16S rRNA gene, which was also quantified by qPCR. The 16S rRNA gene was amplified using the 357-F: 5′-CCTACGGGAGGCAGCAG-3′ and 518-R: 5′-ATTACCGCGGCTGCTGG-3′ primers that were also used to generate the 16S rRNA gene libraries.

4.3.6 Statistical Analysis

Raw data including adapters or low-quality reads would affect the assembly and following analysis. Therefore, in order to obtain high quality clean reads, FASTP (Chen et al., 2018) (version 0.18.0) was used to further filter the original reads according to the following rules. The UPARSE (Edgar et al., 2013) (version 9.2.64) pipeline was used to cluster the effective tags into operational taxonomic units (OTUs) with similarity ≥ 97%. This package was also used to calculate the

Jaccard and Bray–Curtis distance matrix. The α-diversity indexes, such as Chao 1, Shannon, and Simpson, were calculated in QIME (Caporaso et al., 2010) (version1.9.1). The rarefaction analysis was performed using the mothur (Schloss et al., 2009). The alpha index comparison among groups was calculated by Tukey's HSD test and the Kruskal–Wallis H test using the Vegan package in R (Vegan, 2021). Linear discriminant analysis effect size (LEfSe) was used to determine which microorganisms were significantly different among groups (Segata et al., 2011). The antibiotic resistance gene comparison among different periods was statistically analyzed using ANOVA with Tukey's multiple comparison test by SPSS software version 24.0 (SPSS, Inc., Chicago, IL, USA).

4.4 Results

4.4.1 Animal Weight Gain, and 16S rRNA Gene Sequencing Overview

None of the dairy cows in this research received any other antibiotic treatments. The dairy cows were weighed prior to the medication and the recovery period (one quarter was infected; 560–686 kg body weight; 105–226 days in milk; 34.26–39.12 kg of milk per day). The growth rate of the dairy cows was not influenced by the antibiotic medication ($p > 0.05$). The raw reads of sequences per sample ranged from 35,133 to 105,031. After cleaning, the tags of sequences per sample ranged from 33,984 to 102,801. The OTU numbers ranged from 795 to 2344.

4.4.2 α-Diversity

The rarefaction analysis performed for each fecal sequence dataset retrieved rarefaction curves. The result suggested that the sample size was large enough to represent the bacterial diversity present in the communities (Figure 4-2). Microbial diversity within the α diversity was measured by richness (Chao 1) and diversity indices (Shannon and Simpson). The average values of the Shannon diversity index and the average Chao 1 index for the microbiota both decreased significantly at the medication period ($p < 0.05$), suggesting that the total number of species and the abundance of microbiota were all decreased. However, the mean values of the Simpson index did not show significant differences among the different periods (Figure 4-3).

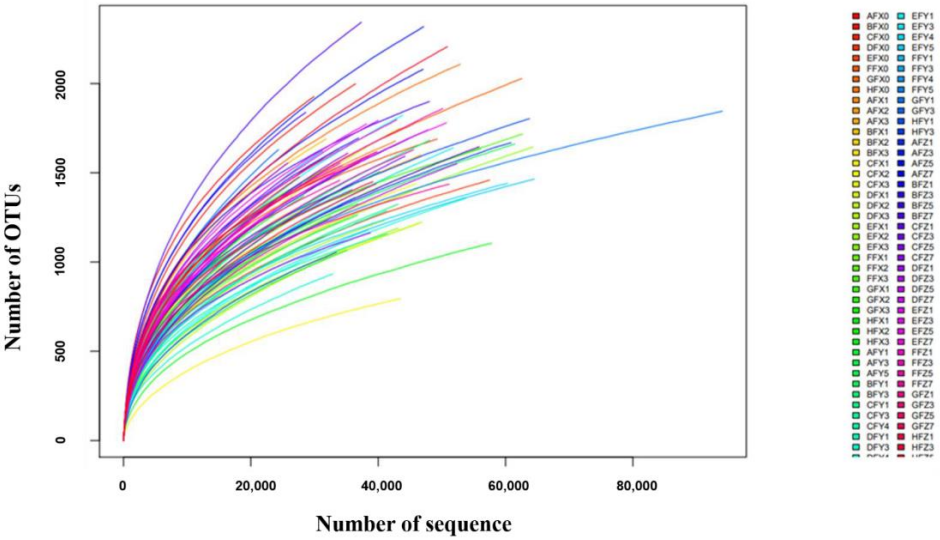
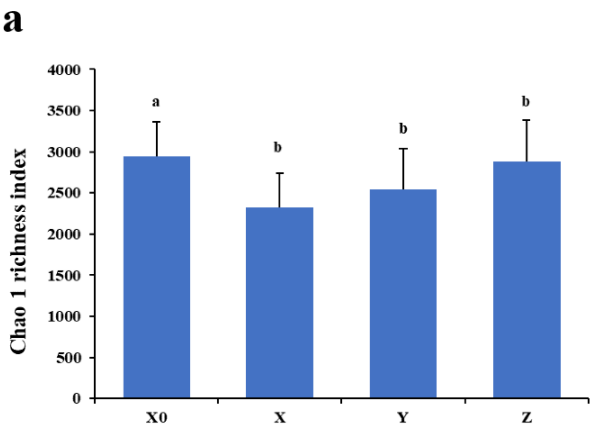


Figure 4-2. The rarefaction analysis performed using mothur. The rarefaction curve based on the species diversity showed sufficient coverage for the sequences. The first letter of the number (A–H) represented the different cows. X0, X (X1, X2, X3), Y (Y1, Y3, Y4, Y5), and Z (Z1, Z3, Z5, Z7) indicate the periods Day 0, medication, withdrawal, and recovery, respectively.



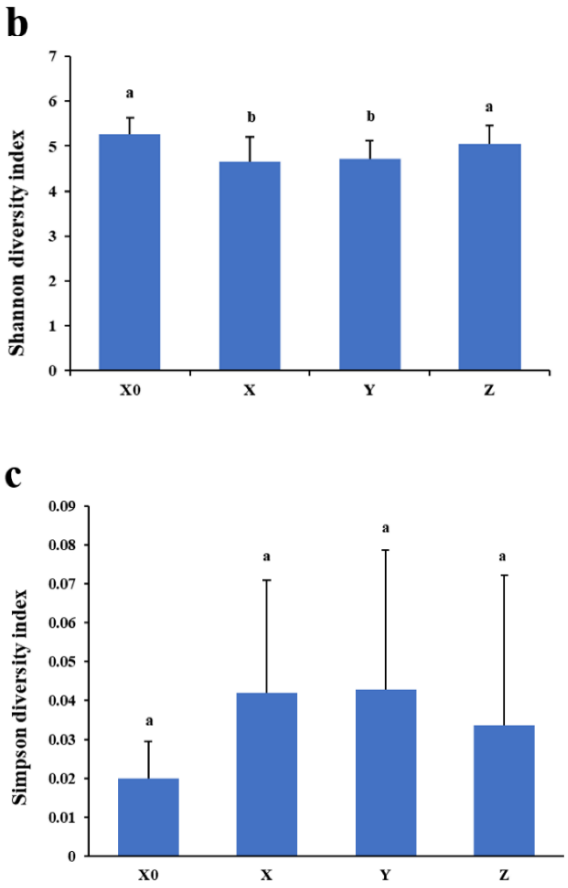


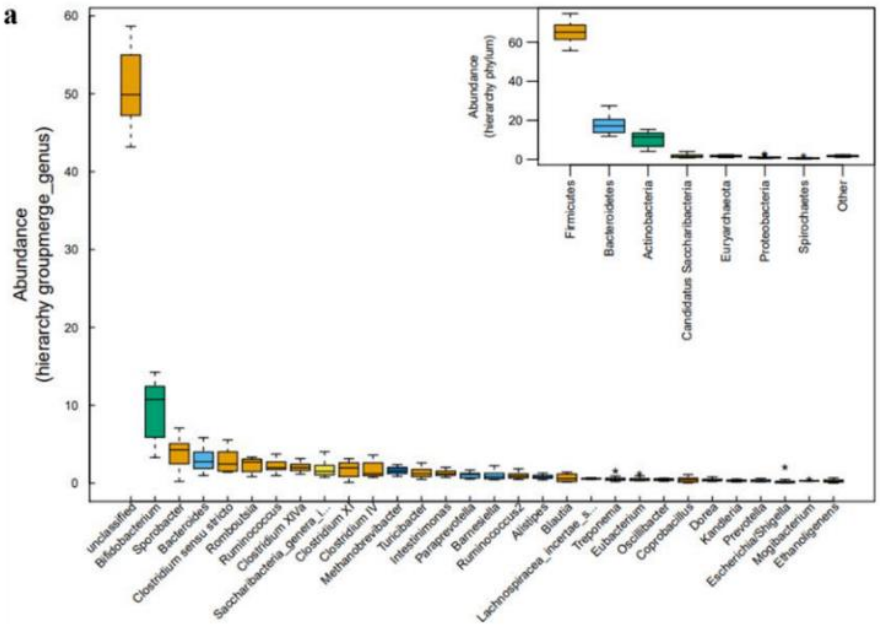
Figure 4-3. (a) Chao 1 richness index of samples from four different periods. (b) Shannon diversity index of samples from four different periods. (c) Simpson diversity index of samples from four different periods. Different lowercase letters within each sampling group represent significantly different means ($p < 0.05$). X0, X, Y, and Z indicate the periods Day 0, medication, withdrawal, and recovery, respectively.

4.4.3 Microbial Community Analysis

The distribution of the most abundant classes and families in the fecal samples are displayed in Figures S1 and S2. At the class level, *Clostridia* was the most abundant bacterium at all four periods, accounting for about 60% of all bacteria,

followed by *Bacteroidia*, *Actinobacteria*, and *Erysipelotrichia*. These four classes accounted for over 85% of all bacteria (Figure S1). At the family level, a total of 50 taxa were detected in the feces. *Ruminococcaceae*, *Lachnospiraceae*, *Bifidobacteriaceae*, and *Porphyromonadaceae* were the four most abundant (Figure S2).

To obtain further insights, the statistical differences in genera in the fecal samples were analyzed using the Vegan R package. At the genus level, *Bifidobacterium*, *Sporobacter*, *Bacteroides*, *Clostridium* sensu stricto, *Romboutsia*, and *Ruminococcus* dominated the fecal samples (Figure 4-4a). *Bifidobacterium* belongs to *Actinobacteria*; *Sporobacter*, *Clostridium* sensu stricto, *Romboutsia*, and *Ruminococcus* belong to *Firmicutes*; and *Bacteroides* belongs to *Bacteroidetes* (Figure 4-4a). *Roseburia* was more abundant when antibiotics were used. The relative abundance of *Bacteroides* (genus), *Bacteroidales* (order), *Bacteroidaceae* (family) and *Bacteroidia* (class) increased with the cephalosporin treatment, and the relative abundance of *Clostridia* (class), *Clostridiales* (order), *Ethanoligenens* (genus), and *Clostridium*_IV (genus) decreased (Figure 4-4b). At the recovery period, the relative abundance of *Clostridium* XI (genus), *Peptostreptococcaceae* (family), *Verrucomicrobiales* (order), *Verrucomicobiae* (class), *Akkermansia* (genus), and *Verrucomicrobiaceae* (family) increased in the fecal samples (Figure 4-4b). Figure S3 displays that the antibiotics were effective in reducing or preventing the growth of *Moraxellaceae*.



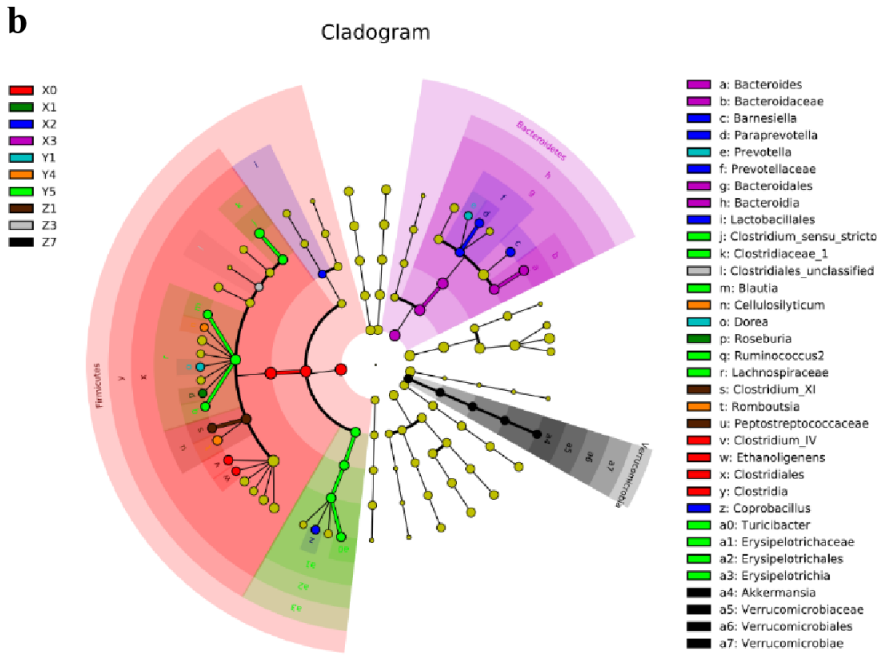


Figure 4-4. Alterations in bacterial genera abundance in the feces at different periods. (a) The difference in abundance of multiple groups of samples after quartile calculation of the abundance of samples at the genus level (b) Lefse analysis phylogenetic tree diagram; different colors in the legend on the left side represent different days in different periods X0 (day 0), X (medication), Y (withdrawal) and Z (recovery). The concentric circles radiating from the outermost to the innermost represent the hierarchical classification levels from phylum to genus (or species). Each small circle at different classification levels represents a taxonomic group at that level, and the diameter of the circle is proportional to its relative abundance. Species without significant differences are colored uniformly in yellow. Red nodes represent microbial groups that play an important role in the red group, while green nodes represent microbial groups that play an important role in the green group. The meaning of other circle colors is similar. The species names represented by English letters in the figure are displayed in the legend on the right side

4.4.4 Antibiotic Resistance Genes

The proportions of sixteen antibiotic resistance genes (*cfx*A, *bla*ROB, *bla*CMY, *mec*A, *bla*CTX-M, *bla*1, and *bla*TEM, *str*A, *str*B, *erm*(A), *erm*(B), *sul*1, *sul*2, *tet*(A), *tet*(B), *tet*(C), *tet*(H) and *tet*(Q), *van*C and *van*G) in fecal samples from four different sampling periods were quantified. Among these 20 resistance genes,

only eight genes (*cfx*A, *bla*TEM, *bla*CMY, *str*B, *tet*(A), *tet*(B), *tet*(C) and *tet*(Q)) showed Cq values during detection in fecal samples. Ceftiofur and cefquinome significantly increased the proportion of *bla*TEM and *cfx*A in the feces at the withdrawal period when compared with Day 0 (Figure 4-5). Other resistance genes were not affected by the antibiotic treatment (data not shown). At the recovery period, the proportion of *cfx*A was significantly decreased compared with the withdrawal period, but the proportion of *bla*TEM was not changed significantly.

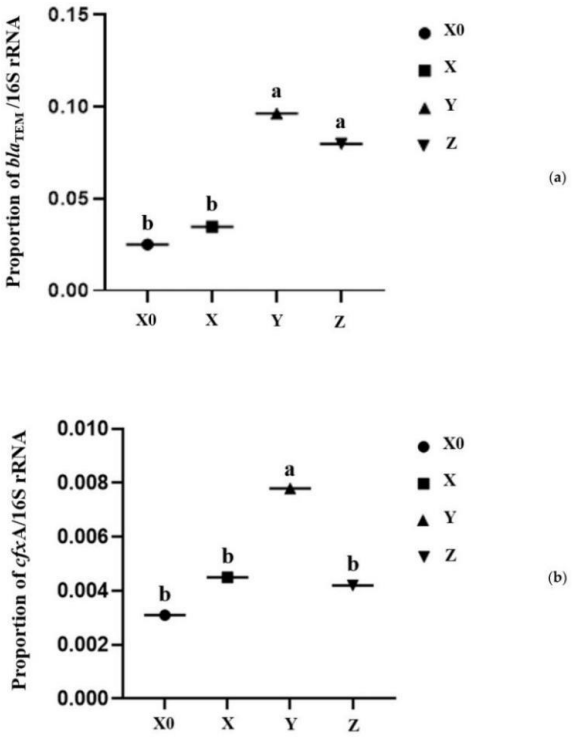


Figure 4-5. Proportion of the antibiotic resistance genes (a) blaTEM and (b) cfxA compared with the 16S rRNA gene. Different lowercase letters suggest significantly different means ($p < 0.05$). X0, X, Y, and Z indicate the periods Day 0, medication, withdrawal, and recovery, respectively.

4.5 Discussion

In our study, the relative abundance of *Bacteroidia* was increased in feces at the medication period. It was reported that the relative abundance of class *Bacteroidia* was increased and the relative abundance of class *Actinobacteria* was decreased

in experimental cattle after 3 days of ceftiofur exposure (Chambers et al., 2015). This was similar to our study. It was also reported that the genus *Bacteroides* strains isolated from human infections contained β-lactamase genes, which reduced sensitivity to antibiotics (Dorado-García et al., 2016). Therefore, we speculated that the increase in *Bacteroidia* at the medication period may be related to the increase of β-lactam resistance genes.

The *bla*TEM has been reported to have a high prevalence in the ceftiofur-resistant bacteria of swine tissues (Chander et al., 2011). In accordance with our previous study, the *bla*TEM in milk that were collected from the cows was significantly increased ($p < 0.05$) after 3 days of cephalosporin exposure (Dong et al., 2021). It was also reported that *bla*TEM was one of the most abundant ARGs in the feces from a pig farm (Gāliņa et al., 2021). In the present study, the β-lactamase encoding genes *bla*TEM and *cfx*A were significantly increased ($p < 0.05$) at the withdrawal period. *cfx*A was considered to be the most abundant β-lactam ARG in ceftiofur-treated cattle feces (Chambers et al., 2015). *cfx*A was also found to be an important gene encoding β-lactamase in *Bacteroides* spp. (García et al., 2008). Avelar et al. (Avelar et al., 2003) detected the *cfx*A gene in 11 *Bacteroides spp*. strains among a total of 73 strains. So, there may be a close correlation between the *Bacteroides* in the feces and the increased genes encoding β-lactam.

The milk microbiota richness of mastitis cows treated with cephalosporins did not decrease (Dong et al., 2021). In the present study, we investigated the effect of cephalosporins on the feces of cows. The results suggested that the cephalosporin treatment indeed affected the abundance of the microbiota in feces, with a decreased richness and decreased diversity, suggesting that antibiotics may have a more pronounced effect on the gut than milk. It may be because the ceftiofur sodium has a pKa value of 3.7 and insufficient lipid-soluble properties to penetrate breast milk (Fernández-Varón et al., 2016). It was reported that when the cattle were treated with ceftiofur, the β-lactam ARGs in feces were increased, and the ceftiofur-resistant *E. coli* isolates from the feces were greater compared to control cattle (Lowrance et al., 2007). In a recent study that used qPCR to detect β-lactam ARGs, they were found to be increased in the feces of cows treated with ceftiofur compared to cows without ceftiofur treatment (Chambers et al., 2015). Considering the proportion of *bla*TEM and *cfx*A in feces during the withdrawal period in this study, it seems that the feces might be an important reservoir of the *bla*TEM and *cfx*A genes. Other studies have also confirmed that animal feces are an important reservoir of ARGs (Dorado-García et al., 2016).

Although none of the cattle received tetracyclines during the period of the study, some antibiotic resistance genes coding for tetracycline resistance (*tet*(W) and *tet*(Q)) were detected but did not significantly change at the different periods (data not shown). This may be because the treatment of a kind of antibiotic can provide selective pressure to maintain other unassociated resistance genes by linking to mobile genetic elements (MGEs) (Enne et al., 2004). The co-transfer of *erm*(B)

and *tet*(M) in the presence of erythromycin has been reported in *Streptococcus* pyogenes isolates (Brenciani et al., 2007), and the colocalization of *mef*A, *aph*A3, *tet*(Q), and IS614 was observed in a transposon of *Bacteroide* (Tauch et al., 2002). It has been reported that MGEs promote the mobilization and spread of ARGs in bacteria. High concentrations of ARGs are considered a risk to public health because the ARGs can transfer from the manure compost, becoming pathogens in agricultural soil (Dorado-García et al., 2016). The resistant bacteria and resistance genes in the feces can also be seen as a serious problem because they may transfer among cattle and result in antibiotic treatment failure. Therefore, the appropriate use of antibiotics in dairy cattle is an important process to avoid the spread of ARBs and ARGs.

4.6 Conclusion

This study provides a snapshot of the changes in the fecal microbiota and resistome affected by cephalosporins. The richness and diversity of the bacterial communities were significantly decreased at the medication period. The relative abundance of *Bacteroides*, *Bacteroidaceae*, *Bacteroidales*, and *Bacteroidia* increased, and the relative abundance of *Clostridia*, *Clostridiales*, *Ethanoligenens*, and *Clostridium* IV decreased at the withdrawal period. This research suggests that cephalosporins had a measurable and immediate effect on the fecal microbiota. However, the cephalosporins increased the proportion of the β-lactam genes *bla*TEM and *cfx*A at the withdrawal period. The long-term (>10 days) effect of the cephalosporin treatment on the fecal microbiota and resistome are worthy of further investigation. At the same time, it is important to develop the appropriate management to control the transfer of ARGs.

4.7 Acknowledgments

This research was funded by the Scientific Research Project for Major Achievements of Agricultural Science and Technology Innovation Program (ASTIP) (No. CAAS-ZDXT2019004), the Agricultural Science and Technology Innovation Program (ASTIP-IAS12), China Agriculture Research System of MOF and MARA, and Project of Risk Assessment on Raw Milk (GJFP2019027).

4.8 Supplemental information

Supplemental information is available in the online version of this article.

4.9 References

Avelar, K. E., Otsuki, K., Vicente, A. C., Vieira, J. M., de Paula, G. R., Domingues, R. M., & Ferreira, M. C. (2003). Presence of the cfxA gene in Bacteroides distasonis. Research in microbiology, 154(5), 369–374.

Bengtsson-Palme, J., and Larsson, D. G. J. (2015). Antibiotic resistance genes in the environment: prioritizing risks. Nat Rev Microbiol.

Brenciani, A., Bacciaglia, A., Vecchi, M., Vitali, L.A., Varaldo, P.E., Giovanetti, E. (2007). Genetic elements carrying erm(B) in Streptococcus pyogenes and association with tet(M) tetracycline resistance gene. Antimicrob Agents Ch.;51(4):1209–16.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., Fierer, N., Peña, A. G., Goodrich, J. K., Gordon, J. I., Huttley, G. A., Kelley, S. T., Knights, D., Koenig, J. E., Ley, R. E., Lozupone, C. A., McDonald, D., Muegge, B. D., Pirrung, M., Reeder, J., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. Nature methods, 7(5), 335–336.

Chambers, L., Yang, Y., Littier, H., Ray, P., Zhang, T., and Pruden, A. (2015). Metagenomic analysis of antibiotic resistance genes in dairy cow feces following therapeutic administration of third generation cephalosporin. Plos One, 10(8).

Chander, Y., Oliveira, S., and Goyal, S.M. (2011). Characterisation of ceftiofur resistance in swine bacterial pathogens. Vet J, 187(1), 139-141.

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics (Oxford, England), 34(17), i884–i890.

Dong, L., Meng, L., Liu, H.M., Wu, H.M., Hu, H.Y., Zheng, N., Wang, J.Q., and Schroyen, M. (2021). Effect of therapeutic administration of β-lactam antibiotics on the bacterial community and antibiotic resistance patterns in milk. J Dairy Sci. 104(6): 7018-7025.

Dorado-García, A., Mevius, D. J., Jacobs, J. J., Van Geijlswijk, I. M., Mouton, J. W., Wagenaar, J. A., & Heederik, D. J. (2016). Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands. The Journal of antimicrobial chemotherapy, 71(12), 3607–3619.

Edgar R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nature methods, 10(10), 996–998.

Enne, V. I., Bennett, P. M., Livermore, D. M., & Hall, L. M. (2004). Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. The Journal of antimicrobial chemotherapy, 53(6), 958–963.

Fang, H., Wang, H., Cai, L., Yu, Y. (2015). Prevalence of antibiotic resistance genes and bacterial pathogens in long-term manured greenhouse soils as revealed by metagenomic survey. Environ Sci Technol.;49(2):1095–104.

Fernández-Varón, E., Cárceles-García, C., Serrano-Rodríguez, J. M., & Cárceles-Rodríguez, C. M. (2016). Pharmacokinetics (PK), pharmacodynamics (PD), and PK-PD integration of ceftiofur after a single intravenous, subcutaneous and subcutaneous-LA administration in lactating goats. BMC veterinary research, 12(1), 232.

Foster, D.M., Jacob, M.E., Farmer, K.A., Callahan, B.J., and Papich, M.G. (2019). Ceftiofur formulation differentially affects the intestinal drug concentration, resistance of fecal Escherichia coli, and the microbiome of steers. PLos ONE, 14(10), e0223378.

García, N., Gutiérrez, G., Lorenzo, M., García, J.E., Píriz, S., Quesada, A. (2008) Genetic determinants for cfxA expression in Bacteroides strains isolated from human infections. J Antimicrob Chemoth. 62: 942–947.

Gāliņa, D., Balins, A., & Valdovska, A. (2021). The Prevalence and Characterization of Fecal Extended-Spectrum-Beta-Lactamase-Producing Escherichia coli Isolated from Pigs on Farms of Different Sizes in Latvia. Antibiotics (Basel, Switzerland), 10(9), 1099.

Guo, C., Liao, X., Wang, M., Wang, F., Yan, C. In vivo pharmacodynamics of Cefquinome in a neutropenic mouse thigh model of Streptococcus suis serotype 2 at varied initial inoculum sizes. Antimicrob Agents Ch. 2016; 60:1114–20.

Halasa, T., Huijps, K., Østerås, O., and Hogeveen, H. (2007). Economic effects of bovine mastitis and mastitis management: a review. Vet Quart, 29(1), 18-31.

Holman, D.B., Yang, W., and Alexander, T. W. (2019). Antibiotic treatment in feedlot cattle: a longitudinal study of the effect of oxytetracycline and tulathromycin on the fecal and nasopharyngeal microbiota. Microbiome, 7(1), 86.

Huang X., Zheng J., Tian S., Liu C., Liu L., Wei L., Fan H., Zhang T., Wang L., Zhu G., Xu K. (2019). Higher temperatures do not always achieve better antibiotic resistance gene removal in anaerobic digestion of swine manure. Appl Environ Microbiol, 22;85(7): e02878-18.

Huijps, K., Lam, T.J., and Hogeveen, H. (2008). Costs of mastitis: facts and perception. J Dairy Res, 75(1), 113-120.

Kaniyamattam, K., Vries, A.D., Tauer, L.W., and Grhn, Y.T. (2020). Economics of reducing antibiotic usage for clinical mastitis and metritis through genomic selection - sciencedirect. J Dairy Sci, 103(1), 473-491.

Kuipers, A., Koops, W. J., and Wemmenhove, H. (2016). Antibiotic use in dairy herds in the Netherlands from 2005 to 2012. J. Dairy Sci. 99:1632–1648.

Li, Y., Feng, B., Gu, X., Yang, D., Zeng, Z., Zhang, B., and Ding, H. (2016). Correlation of PK/PD Indices with Resistance Selection for Cefquinome against Staphylococcus aureus in an In Vitro Model. Frontiers in Microbiology, 7, 466.

Liu, J.H., Wei, S.Y., Ma, J.Y., Zeng, Z.L., Lü, D.H., Yang, G.X., Chen, Z.L. (2007). Detection and characterization of CTX-M and CMY-2 b-lactamases among Escherichia coli isolates from farm animals in Guangdong province of China. Int J Antimicrob Ag 29, 576–581.

Lowrance, T.C., Loneragan, G. H., Kunze, D.J., Platt, T. M., Ives, S. E., and Scott, H. M., Norby, B., Vet, C.M., Echeverry, A., Brashears, M.M. (2007). Changes in antimicrobial susceptibility in a population of Escherichia coli isolated from feedlot cattle administered ceftiofur crystalline-free acid. American Journal of Veterinary Research, 68(5), 501.

Pehlivanoglu F., Turutoglu H., Ozturk D. (2016). CTX-M-15-Type Extended-Spectrum Beta-Lactamase-Producing Escherichia coli as Causative Agent of Bovine Mastitis. Foodborne Pathog Dis. Sep;13(9):477-82.

Santos, J.E., Cerri, R.L., Ballou, M. A., Higginbotham, G. E., and Kirk, J. H. (2004). Effect of timing of first clinical mastitis occurrence on lactational and reproductive performance of holstein dairy cows. Anim Reprod Sci. 80(1-2), 31-45.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J., & Weber, C. F. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and environmental microbiology, 75(23), 7537–7541.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. Genome biology, 12(6), R60. https://doi.org/10.1186/gb-2011-12-6-r60

Stannarius, C., Bürgi, E., Regula, G., Zychowska, M. A., and Stephan, R. (2009). Antimicrobial resistance in Escherichia coli strains isolated from swiss weaned pigs and sows. Schweiz Arch Tierheilkd, 151(3), 119-125.

Su, J.Q., Wei, B., Ou-Yang, W.Y., Huang, F.Y., Zhao, Y., and Xu, H.J. (2015). Antibiotic resistome and its association with bacterial communities during sewage sludge composting. Environ Sci Technol, 49(12), 7356-63.

Tauch, A., Götker, S., Pühler, A., Kalinowski, J., Thierbach, G. (2002). The 27.8-kb R-plasmid pTET3 from Corynebacterium glutamicum encodes the aminoglycoside adenyltransferase gene cassette aadA9 and the regulated tetracycline efflux system tet33 flanked by active copies of the widespread insertion sequence IS6100. Plasmid 48:117–129.

Turk, R., Piras, C., Kovai, M., Samardžija, M., Ahmed, H., and Canio, M. D., Urbani, A., Meštrić, Z.F., Soggiu, A., Bonizzi, L., Roncada, P. (2012). Proteomics of inflammatory and oxidative stress response in cows with subclinical and clinical mastitis. J Proteomics, 75(14).

USDA. Dairy. 2014. Milk Quality, Milking Procedures, and Mastitis in the United States. Fort Collins: USDA–APHIS–VS–CEAH–NAHMS; 2016.

Vegan: Community Ecology Package, R Package Version 1.17-4. Available online: https://cran.r-project.org/web/packages/vegan/index.html (accessed on 31 December 2021).

Venturini, C., Bowring, B., Fajardo-Lubian, A., Devine, C., and Iredell, J. (2020). Effects of antibiotic treatment with piperacillin/tazobactam versus ceftriaxone on the composition of the murine gut microbiota. Antimicrob Agents Ch, 65(2).

Wang, J., Shan, Q., Ding, H., Liang, C., Zeng, Z. (2014). Pharmacodynamics of cefquinome in a neutropenic mouse thigh model of Staphylococcus aureus infection. Antimicrob Agents Ch. 58:3008–12.

Xiong, W., Wang, Y., Sun, Y., Ma, L., Zeng, Q., Jiang, X., Li, A., Zeng, Z., Zhang, T. (2018). Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. Microbiome, 6(1), 34.

Zhao, S., White, D.G., Mcdermott, P.F., Friedman, S., English, L., and Ayers, S., Ayers, S., Meng, J., Maurer, J.J., Holland, R., Waker, R.D. (2001). Identification and expression of cephamycinase blacmy genes in Escherichia coli and Salmonella

isolates from food animals and ground meat. Antimicrob Agents Chemother, 45(12), 3647-3650.

Zhou, Y., Zhao, D., Yu, Y., Yang, X., Shi, W., Peng, Y. (2015). Pharmacokinetics, bioavailability and PK/PD relationship of cefquinome for Escherichia coli in beagle dogs. J Vet Pharmacol Ther. 38:543–8

Chapter 5

The study on the heteroresistance to cephalosporins of Staphylococcus in milk from mastitis suffering cows

Chapter 5. The study on the heteroresistance to cephalosporins of Staphylococcus in milk from mastitis suffering cows

Lei Dong,[1,2,3*] Lu Meng,[1,2*] Huimin Liu,[1,2] Nan Zheng,[1,2] Martine Schroyen[3] and Jiaqi Wang,[1,2†]

[1]Key Laboratory of Quality and Safety Control for Milk and Dairy Products of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[2]Laboratory of Quality and Safety Risk Assessment for Dairy Products of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[3]Precision Livestock and Nutrition Laboratory, Teaching and Research Centre (TERRA), Gembloux Agro-Bio Tech, University of Liège, Gembloux 5030, Belgium

[*] Authors who contribute equally to this work.

[*]Correspondence: jiaqiwang@vip.163.com

Running title: Heteroresistance to cephalosporins of *Staphylococcus* in milk

5.1 Abstract

Heteroresistance can bring about therapy failure and is hard to detect by routine methods in the laboratories. In this study, we studied the prevalence of ceftiofur heteroresistant *Staphylococcus* isolates. In total, 151 *Staphylococcus* isolates were collected from milk from mastitis suffering cows from the major dairy-production areas of China. The disk diffusion method and population analysis profiling (PAP) method were carried out to evaluate the existence of heteroresistance. The heteroresistance stability was investigated by culturing isolates for seven days in ceftiofur-free medium. Whole-genome sequencing analysis was used to identify the nucleotide and resultant protein sequences. Among the isolates, 15 strains (15/151, 9.9%) showed heteroresistance by the disk diffusion method, and, of those 15, three strains (3/15, 20%) exhibited heteroresistant phenotypes by the PAP method. Two of these heteroresistant strains were unstable, as the minimal inhibitory concentrations (MICs) decreased after 1-week daily culture. Whole-genome sequencing displayed that, for strains with heteroresistant phenotypes, there were single nucleotide polymorphisms in the *mecA* gene, leading to different protein sequences, which might be associated with ceftiofur heteroresistance. There were two extra mutations in the heteroresistant stable isolate (D12-4), which might have resulted in the formation of a stable resistant subpopulation in heteroresistant *Staphylococcus*. These findings improve knowledges about the emergence of ceftiofur-heteroresistant *Staphylococcus* isolates and the application of ceftiofur as therapy for the treatment for mastitis in dairy cows.

Keywords: *Staphylococcus*, ceftiofur heteroresistance, milk, *mecA*

5.2 Introduction

Staphylococcus spp. strains are commonly found in the all kind of natural environment such as water, soil, and air. They are also an integral component of the microbiota of the skin and mucous membranes of humans and some animals. In humans, *Staphylococcus* is associated with skin infections, pneumonia and endocarditis (Donadu et al., 2022). Also in livestock animals, *Staphylococcus* can cause important infections, and can be spread from animals to humans (Fluit et al., 2012; Tegegne et al., 2021). In addition, S*taphylococcus* exist in foods such as fermented meat products, milk, and cheese (Milala et al., 2021). Several *Staphylococcus spp*. strains can produce enterotoxins, which are often associated with coagulase-positive species such as *Staphylococcus aureus* (*S. aureus*). Coagulase-negative *Staphylococcus* strains have been found containing enterotoxin-related genes and are also capable of producing these enterotoxins, which have been described as the causative agent of foodborne disease outbreaks (Casaes et al., 2016; da Silva Cândido et al.,2020).

Ceftiofur is a kind of third-generation cephalosporin antibiotic with broad-

spectrum antibacterial activity, commonly used in the treatment of infectious diseases in dairy cows (Saini et al., Dong et al., 2021). It has been reported that 80% of *S. aureus* and 97.5 % of *S. chromogens* isolates from milk samples of mammary quarters of cows were classified as intermediate, while the remaining isolates (20% of *S. aureus* and 2.5% of *S. chromogenes*) were all resistant to ceftiofur (Leite et al., 2018).

Resistant bacteria are the main reason for failure of antibiotic treatment and they lead to an increased mortality after infection (Nicoloff et al., 2019). However, even if bacteria have been identified as sensitive to antibiotics, antibiotics are not always effective in treating bacterial infection diseases (European Committee on Antimicrobial Susceptibility Testing, 2019). The main reason is that a specific population of pathogens contains subpopulations of these pathogens with lower susceptibility to the antibiotic than the dominant population and thus the population can exhibit phenotypic heterogeneity, which makes it difficult to categorize bacteria as sensitive or resistant (Andersson et al., 2019). This phenomenon is entitled antibiotic heteroresistance. Several studies have reported that heteroresitant bacteria may lead to failure of antibiotic treatment (Pournaras et al., 2005; Kelley et al., 2011). According to some previous studies, antibiotic heteroresistance is defined as the existence of a subpopulation of cells capable of at least 8-fold higher antibiotic concentrations than the highest concentration that does not inhibit the growth of the dominant population (El-Halfawy et al., 2015). Heteroresistance is considered to be a crucial intermediate stage before the development of full resistance (Morand et al., 2007). Heteroresistance, which describes the presence or emergence of resistant subpopulations, is a great challenge for the microbiology field. Falsely identified and interpreted susceptible microorganisms can have a bad effect on the treatment, resulting in higher morbidity, mortality, and overuse of second-line and last-line antibiotics. Therefore, the detection and characterization of heteroresistant isolates is crucial for maximizing the antibiotics efficiency.

Almost all research on heteroresistance have been performed in human rather than animal isolates. Several studies on heteroresistance to cephalosporin have been reported in *Acinetobacter baumannii* (Hung et al., 2012), *Enterobacteriaceae* (Søgaard et al., 1985), *S. aureus* (Saravolatz et al., 2014), and *Escherichia coli* (Ma et al., 2016), however, most of them are concerned with Gram-negative bacteria. So far, no study has screened for heteroresistance to ceftiofur in *Staphylococcus spp*., from milk. Therefore, the aims of this study were to screen the heteroresistant bacteria and explore the mechanisms of ceftiofur heteroresistance in *Staphylococcus spp*. isolates obtained from raw milk. Our study is the first study to research the mechanism of heteroresistance *Staphylococcus* to ceftiofur in raw milk, providing a basis for reducing the development and spread of heteroresistance.

5.3 Materials and methods

5.3.1 Bacterial isolates

A total of 151 *Staphylococcus spp*. strains studied in the present study were isolated from milk samples from mastitis suffering cows (One quarter was infected; 545–690 kg body weight; 99–246 days in milk; 33.12–40.58 kg of milk per day. All cows received ceftiofur during illness) from the major dairy-production areas of China, with 31 *S. aureus* from Xinjiang, 70 *Staphylococcus spp*. from Tianjin, 9 *S. aureus* from Qingdao, and 41 *S. aureus* from Inner Mongolia. The isolates were identified as *Staphylococcus spp*. by polymerase chain reaction (PCR) (Bio-Rad S1000, United States) and 16S rRNA sequencing. All isolates were stored at -80℃ until further testing. Frozen isolates were thawed and sub-cultured at least twice prior to treatment. The strains were cultured on nutrient agar (Thermo Fisher Oxoid, Basingstoke, UK) at 37℃.

5.3.2 Antimicrobial susceptibility testing

The susceptibility determinations of clinical routine antibiotics, including penicillin (PEN), ampicillin (AMP), amoxicillin and clavulanate potassium (ACP), oxacillin (OXA), cephalothin (CEP), ceftiofur (CEF), erythromycin (ERY), clindamycin (CLI), gentamicin (GEN), doxycycline (DOX), florfenicol (FLO), rifaximin (RIF), vancomycin (VAN), sulfisoxazole (SUL), pediatric compound sulfamethoxazole tablets (PCST), ciprofloxacin (CIP), were assayed in triplicate for each bacterial strain by a broth microdilution method (BMD) and interpreted in line with CLSI guidelines (Clinical and Laboratory Standards Institute, 2018). *S. aureus* ATCC 29213 was used as a quality control strain. The multiple antibiotic resistance (MAR) index for each bacterium was measured against 16 antimicrobial agents according to the method of Blasco et al. (Blasco et al., 2008). The MAR index is a reasonable risk evaluation method. A MAR index value of 0.20 is used as threshold to distinguish the low-risk (<0.20) and high-risk (>0.20) (Krumperman et al., 1983).

$$\text{MAR index} = \frac{\text{number of antibiotics to which isolate was resistant}}{\text{total number of antibiotics tested}}$$

5.3.3 Primary screening of heteroresistant bacteria

Hereroresistance to CEF was detected by the disc diffusion method on Mueller-Hinton agar (MHA) (Thermo Fisher Oxoid, Basingstoke, UK) using a 0.5 McFarland standard bacterial suspension and paper discs containing CEF concentrations of 10 μg (Jinzhang, Tianjin, China). Following incubation at 37℃ for 24h, the presence of distinct colonies growing within a well-defined inhibition zone was deemed an indication of CEF-heteroresistant *Staphylococcus* (CHS), while isolates displaying inhibition halos but no populations were defined as non-CHS.

5.3.4 Population analysis profiling

After the primary screening, 15 non-duplicate isolates that showed heteroresistance were further screened using the population analysis profile (PAP) methods according to the previously published study with some modifications

(Hong et al., 2020). Each isolate represented a single milk sample. First, after overnight growth on MHA plates, one bacterial colony was inoculated into fresh MH broth while shaking at 37℃ for 24 hours. A single colony of each isolate was regulated to 10^8 CFU/mL by the 0.5 McFarland standard bacterial suspension. The serial dilutions of the bacterial suspension (10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}) were carried out in saline. Then, a 100 μL aliquot of 10^8 CFU/mL-10^3 CFU/mL bacterial suspension was spread onto MHA plates containing either 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 mg/mL CEF. The limit of quantification (LOQ) was 20 CFU/mL. The PAP method was duplicated with three independent biological repeats.

5.3.5 Stability of resistant subpopulations

The stability test was conducted to study if the resistant subpopulations preserved their CEF resistance phenotype. Two to three colonies were selected from the PAP test plates containing heteroresistant *Staphylococcus spp*. strains. The resistant subpopulations were serially inoculated for seven extra overnight incubation periods (about 10 generations each day) in the absence and presence of CEF. Then, the MIC of CEF were determined by the broth microdilution method, and the resistant subpopulation was considered unstable if the MICs decreased or recovered to that of the parental strain. All measurements were performed with three independent biological replicates.

5.3.6 Whole-genome sequence

The genome of nine *Staphylococcus* strains [three *Staphylococcus* strains were identified heteroresistant to ceftiofur by both the disc diffusion method as well as the PAP method (HR*HR), three *Staphylococcus* strains identified heteroresistant to CEF by the disc diffusion method but non-heteroresistant by the PAP method (HR*S), and three *Staphyloccocus* strains were sensitive to CEF (S)] were sequenced at the Beijing Genomics Institute (Shenzhen, China) using both the PacBio RS II and Illumina HiSeq 4000 platforms. For the PacBio platform, four SMRT cells Zero-Mode Waveguide arrays were used to generate the subreads set. Subreads with a length less than 1 kb were deleted. The Pbdagcon program (https://github.com/PacificBiosciences/pbdagcon) was utilized for self-correction. Draft genomic units were assembled using the Celera Assembler against a high-quality corrected set of circular consensus subreads. To enhance the accuracy of the genome sequences, single-base corrections were performed using GATK (https://www.broadinstitute.org/gatk/) and SOAP tool packages (SOAP2, SOAPsnp, SOAPindel). The filtered Illumina reads were then mapped to the bacterial plasmid database (http://www.ebi.ac.uk/genomes/plasmid.html, last accessed July 8, 2016) using SOAP to identify any plasmids that may have emerged. Gene prediction was performed on the *staphylococcus* genome assembly by glimmer3 (http://www.cbcb.umd.edu/software/glimmer/) with Hidden Markov models. Resistance gene were identified according to the core dataset in ARDB (Antibiotic Resistance Genes Database) database. Single nucleotide variants analysis and protein sequences were identified by whole-

genome alignment using the MegAlign software package.

5.4 Results

5.4.1 Antibiotic susceptibility results of *Staphylococcus*

The antibiotic resistance for different kinds of antibiotics in the 151 tested strains of *Staphylococcus* are listed in Table 5-1 and Figure 5-1. The antibiotic susceptibility results of the 151 *Staphylococcus* isolates are listed in Supplementary Table S1. In addition, the 151 tested strains had a MAR index value ranging from 0 to 0.375 (Supplemental Table S2), with 18.5% (28/151) of isolates having MAR values > 0.20.

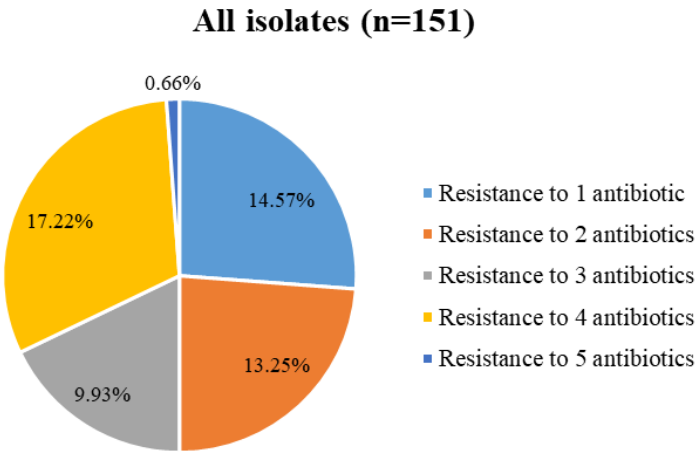


Figure 5-1. Distribution of resistance patterns.

Table 5-1 The resistance rate of different antibiotics in 151 Staphylococcus

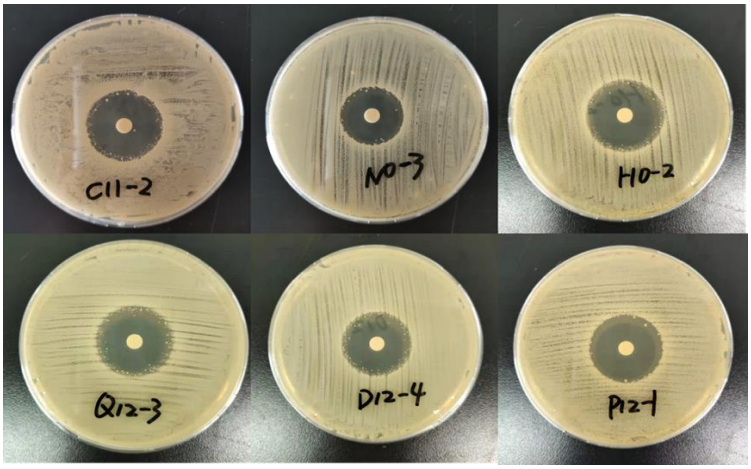
Antibiotics	No. of Resistance isolates (%)
Ampicillin	58 (38.41%)
Erythromycin	54 (35.76%)

Clindamycin	32 (21.19%)
Sulfisoxazole	26 (17.22%)
Amoxicillin/clavulanic acid	23 (15.23%)
Oxacillin	9 (5.96%)
Ceftiofur	8 (5.30%)
Ciprofloxacin	5 (3.31%)
Gentamicin	4 (2.65%)
Trimethoprim/sulfamethoxazole	2 (1.32%)
Doxycycline	0 (0%)
Vancomycin	0 (0%)
Penicillin	0 (0%)
cephalothin	0 (0%)
Florfenicol	0 (0%)
Rifampin	0 (0%)

5.4.2 Verification of CEF heteroresistance among *Staphylococcus* isolates

The CEF heteroresistance among *Staphylococcus* was identified performing the disc diffusion method and the PAP method. Among the 151 isolates screened by the disc diffusion method, we found scattered colonies in the inhibition zones around the CEF disks in 15 isolates (Figure 5-2a), showing the potential to induce resistance of CEF under the pressure of CEF. The PAP method confirmed the presence of a heteroresistant subpopulation in 20.0% (3/15) of the strains that grew to 4 or 8 mg/L CEF from milk samples from mastitis suffering cows of Tianjin city (Figure 5-2b).

a



b

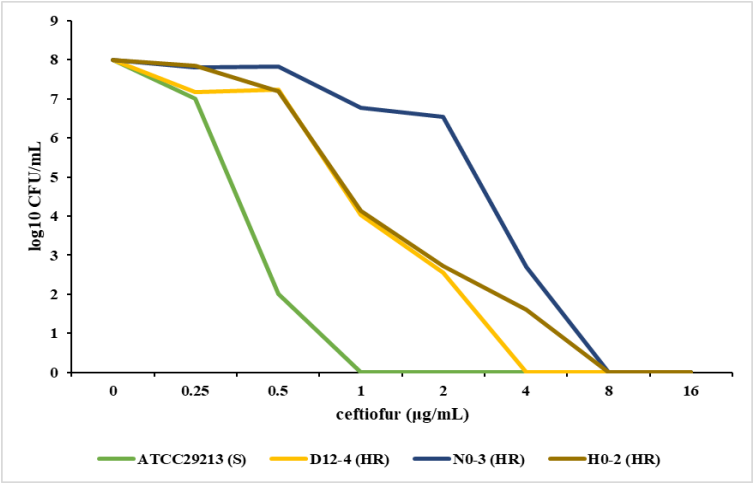


Figure 5-2. Verification of CEF heteroresistance among Staphylococcus strains. (a) Heteroresistance apperance of Staphylococcus isolates to CEF identified by the disk diffusion method. (b) PAP method of the CEF heteroresistant strains and the control strain (ATCC 29213)

5.4.3 Stability of the subpopulations of CEF-heteroresistant *Staphylococcus*

We confirmed that three *Staphylococcus* isolates (D12-4, H0-2, N0-3) showed CEF heteroresistance, and it was observed that H0-2 and N0-3 were susceptible to all tested antibiotics, while D12-4 was resistant to AMP but susceptible to CEF (Table S1). The single phenotypes and PAP frequencies of these subpopulations from parental isolates are shown in Table 5-2. The results demonstrated that H0-2 and N0-3 were unstable and transient without other antibiotic exposure after seven days of serial subculturing. The D12-4 maintained the resistance to CEF after seven days.

Table 5-2. Characteristics of 3 CEF-heteroresistant Staphylococcus isolates included in this

Isolate	BMIC (mg/L)	HCG in PAPs (mg/L)	PAP	MIC after 1-week[a]	HP	PAP frequency[b]
D12-4	0.25	4	Heteroresistant	4	Stable	2.72×10^{-7}
H0-2	0.25	8	Heteroresistant	2	Unstable	2.44×10^{-6}
N0-3	0.5	8	Heteroresistant	4	Unstable	2.63×10^{-7}

[a] Resistant colonies MIC after 1-week daily passages onto CEF free medium

[b]The frequency of the appearance of CEF-heteroresistant subpopulation was calculated according to the following formula: [number of colonies on CEF (2, 4 μg/mL) dish × dilution]/ (number of colonies on CEF-free dish × dilution.

Abbreviation: BMIC, Broth MIC; HCG, Highest concentration of growth; HP, Heterogeneous phenotype

5.4.4 Whole-genome sequences, general genomic features, and protein sequences

In our study, the complete genomic sequences of three kinds of *Staphylococcus* strains were determined. The D12-4, H0-2, and N0-3 were heteroresistant to CEF by both the disc diffusion method as well as the PAP method (HR*HR), the C8-2, C11-2, and Q12-3 were heteroresistant to CEF by disc diffusion method but sensitive to ceftiofur by PAP method (HR*S), and the H0-1, D12-5, C11-1 were sensitive to the CEF (S). The general informations of the nine *Staphylococcus* strains are displayed in Table 5-3. The genomic features of the examined strains are shown in Table S3.

Overall, 18 resistance determinants, showing resistant to 11 classes of

antibiotics, were found in the nine isolates, and all nine isolates carried resistance determinants. All nine *Staphylococcus* strains carried the *vanRE*, *vanRG*, and *vanRD*, *lmrB*, *ykkC*, *ykkD*. All HR*HR strains (D12-4, H0-2, N0-3) carried the β-lactam resistance determinants *mecA* or *bl2a_pc*. Two HR*S strains (C8-2, C11-2) and one S strain (D12-5) carried the β-lactam resistance determinant *mecA*. The *mphC* gene, showing resistance to macrolide, was also detected in all HR*HR strains (D12-4, H0-2, N0-3), two HR*S strains (C8-2, C11-2) and one S strain (D12-5). Only one HR*HR strain (N0-3) carried the *fosB* and *tmrB* gene. However, none of the HR*HR strains (D12-4, H0-2, N0-3) carried genes conferring resistance to trimethoprim, tetracycline, or aminoglycoside.

The presence of the carbohydrate-active enzyme was shown in Table 5-3, it is worth noting that the SpsG was only detected in some HR*HR (D12-4, N0-3) and HR*S strains (C8-2, C11-2).

We also identified the different protein sequences (Table 5-4). The protein sequences differences were detected with regard to the *mecA* gene in six *Staphylococcus* strains. Comparing these strains with the translation of the *mecA1* gene in Staphylococcus sciuri ATCC 29062 (BioProject: PRJNA313047), resulted in 17 differences in D12-4 (HR*HR, stable). There were the same protein sequence differences in 15 positions in the *mecA* gene in H0-2 (HR*HR), C11-2 (HR*S), and D12-5 (S). We also found protein sequence differences in 15 positions in the *mecA* gene in C8-2 (HR*S).

Table 5-3. Resistance phenotypes, distribution of antibiotic genes, and carbohydrate-active enzyme presence in nine Staphylococcus sequenced by whole-genome sequences.

			Resistance determinant											
Strains	**Genus**	**State**	**Glycopeptides**	**polypeptide**	**Lincosamide**	**SMR**	**β-Lactam**	**Macrolide**	**Fosfomycin**	**Tunicamycin**	**Trimethoprim**	**Tetracycline**	**Aminoglycoside**	**Carbohydrate-active enzyme**
D12-4	*Staphylococcus sciuri*	HR* HR[a] stable	*vanXYE, vanRE, vanRG, vanRD*	*bacA*	*lmrB* (3),	*ykkD, ykkC,*	*mecA*	*mphC*						SpsG
H0-2	*Staphylococcus sciuri*	HR* HR	vanXE, *vanRD, vanRG, vanRE*	*bacA*	*lmrB* (4),	*ykkC, ykkD*	*mecA*	*mphC*						
N0-3	*Staphylococcus*	HR* HR	*vanXYE, vanRE*	*bacA*	*lmrB* (4)	*ykkC,*	*mecA*,	*mphC*	*fosB*	*tmrB*				SpsG

	sciuri		(2), *vanRG*, *vanRD*,			*ykkD*	*bl2a_pc*,							
C8-2	*Staphylococcus sciuri*	HR*S[b]	*vanRD*, *vanRG*, *vanRE*, *vanXYE*	*bacA*	*lmrB* (3)	*ykkC*, *ykkD*	*mecA*, *bl2a_pc*,	*mphC*						SpsG
C11-2	*Staphylococcus sciuri*	HR*S	*vanRD*, *vanRG*, *vanRE*, *vanXYE*	*bacA*	*lmrB* (3)	*ykkC*, *ykkD*	*mecA*	*mphC*						SpsG
Q12-3	*Staphylococcus chromogenes*	HR*S	*vanRG*, *vanRA*, *vanRD*, *vanRE*	*bacA*, *bcrA*	*lmrB* (2)	*ykkC*, *ykkD*					dfra20	tet38		
H0-1	*Staphylococcus*	S[c]	*vanRD*, *vanRA*,	*bacA*, *bcrA*	*lmrB* (2)	*ykkC*,					dfra20	tet38	vatd	

	chromogens		*vanRE, vanRG*			*ykkD*								
D12-5	*Staphylococcus sciuri*	S	*vanRE, vanXYE, vanRD, vanRG*	*bacA*	*lmrB* (4)	*ykkC, ykkD*	*mecA*	*mphC*			dfra20			
C11-1	*Staphylococcus chromogens*	S	*vanRD, vanRA, vanRG, vanRE*	*bacA, bcrA*	*lmrB* (2)	*ykkC, ykkD*						tet38	vatd	

[a] The strains were determined as heteroresistance strains screened by the disc diffusion method and the PAP method.

[b] The strains were determined as heteroresistance strains screened by the disc diffusion method but sensitive strains by the PAP method.

[c] The strains were determined sensitive strains screened by the disc diffusion method.

Table 5-4. Nucleotide and protein sequences translation of mecA gene in six Staphylococcus strains.

	Protein sequences					
Gene	**D12-4 (HR*HR)**	**H0-2 (HR*HR)**	**N0-3 (HR*HR)**	**C8-2 (HR*S)**	**C11-2 (HR*S)**	**D12-5 (S)**
mecA	S 18 G R 109 H N 125 D A 130 V D 207 Y Q 241 E L 244 F D 289 E N 304 Q K 313 E I 317 K Q 351 K S 374 N S 408 A L 412 S D 621 N I 625 M	S 18 G R 109 H N 125 D A 130 V Q 241 E L 244 F D 289 E N 304 Q K 313 E I 317 K Q 351 K S 374 N S 408 A D 621 N I 625 M	1-8 lack I 10 V A 14 V G 16 A V 19 I D 26 N K 27 S K 28 Q E 31 D I 33 L N 47 D M 61 I D 76 A N 125 D A 130 V K 136 Q T 200 A D 218 N L 223 T N 225 R	T 62 I L 124 F N 125 D A 130 V V 159 I Q 241 E L 244 F D 289 E N 304 Q K 313 E I 317 K S 374 N S 408 A D 621 N I 625 M	S 18 G R 109 H N 125 D A 130 V Q 241 E L 244 F D 289 E N 304 Q K 313 E I 317 K Q 351 K S 374 N S 408 A D 621 N I 625 M	S 18 G R 109 H N 125 D A 130 V Q 241 E L 244 F D 289 E N 304 Q K 313 E I 317 K Q 351 K S 374 N S 408 A D 621 N I 625 M

			S 234 T I 317 K A 328 S S 408 A G 419 D D 619 N D 621 N I 625 M R 663 N			

5.5 Discussion

According to the World Health Organization (WHO), ceftiofur, a main registered third-generation cephalosporin, is a veterinary critically important antimicrobial and antibiotic (WHO, 2017). Ceftiofur was frequently used as a common antibiotic in the dairy farms from which our samples were obtained (Dong et al., 2021). During the research, we found that a few dairy cows had recurrent mastitis infections. However, only 8 (8/151, 5.30%) strains were resistant to ceftiofur in the antibiotic susceptibility results of *Staphylococcus*, which showed that there may be the appearance of the heteroresistance phenomenon.

In our study, we chose to study *Staphylococcus spp*. since it was the bacteria with the highest isolation rate in milk samples from mastitis suffering cows compared to other common mastitis-associated pathogens such as Enterobacter, Streptococcus, *Klebsiella pneumoniae* (data not shown). However, there are almost no reports on heteroresistance to ceftiofur in *Staphylococcus spp*. in food. As far as we know, this is the first time to investigate the heteroresistance to ceftiofur in *Staphylococcus spp*. in milk.

In this study, we investigated ceftiofur heteroresistance in *Staphylococcus* isolates from milk. There were 15 isolates showing the possibility of inducible resistance to CEF using the disc diffusion method. Only three *Staphylococcus* strains that were determined to be sensitive to ceftiofur were subsequently identified as ceftiofur heteroresistant by the PAP method, which is considered an appropriate method for determining heteroresistance. The prevalence of heteroresistant *Staphylococcus* in our study was lower than that in another study on this subject (Saravolatz et al., 2014). We can think of two main reasons to explain this phenomenon. The first lies in the resistance rate of these strains. In our study, the prevalence of antibiotic resistance (the strains were less than 50% resistant to every antibiotic tested) and multidrug resistance (with 18.5% of isolates having MAR indices >0.20) were both relatively lower than that in previous studies (Odjadjare et al., 2012; Tan et al., 2020), which may have a positive correlation to HR. The other reason is the different sources of the isolates. The strains in our study were collected from mastitis milk, but the *Staphylococcus* strains investigated in some previous reports were collected from people (Ma et al., 2016). In the present study, we found that only one strain was stable but the other two subpopulations were unstable and transient, and the MIC reverted to a lower level. Some previous studies have shown that heteroresistance could often exhibit different MIC levels and stability due to the subpopulation and its mutants (Nicoloff et al., 2019). These results were consistent with the observation in our research. This showed that the routinely performed analysis of strains in the laboratories, might not always completely characterize all bacteria isolated from all kinds of samples. A number of heteroresistance strains were unstable in some previous studies (Nicoloff et al., 2019). It is reported that resistant subpopulations of *Enterobacter cloacae* have increased from less than 10% to more than 80%

following colistin exposure. However, this occurrence was reversible, and strains could recover to the sensitivity levels of pretreatment after being cultured in colistin-free medium. This kind of heteroresistance is hard to detect due to the instability, which is often lost when the antibiotic pressure becomes lower. Moreover, it is difficult to detect with a traditional method, so that strains with a resistant subpopulation being misdiagnosed as sensitive is common, and finally results in treatment failure.

In recent years, more and more studies have shown that point mutations, insertions, and small deletions in genes can be related to antibiotic resistance (Andersson et al., 2019). Recently, it has been reported that the high frequency of heteroresistance in all kinds of bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae*, *S. typhimurium*, and *Acinetobacter baumannii*, were mainly created by unstable, spontaneous tandem amplification of all kinds of genes in subpopulations, finally leading to heteroresistance (Nicoloff et al., 2019). In this study, there were several mutations identified in the β-lactam gene *mecA*. The *mecA* gene is a penicillin binding protein and has a low affinity to β-lactam and catalyzes a penicillin-insensitive transpeptidation. The altered PBP2a production encoded by *mecA*, leading to a low affinity to all β-lactams and endues extensive resistance to β-lactams antibiotics (including cephalosporins and carbapenems), which is not influenced by β-lactamase inhibitors. To our knowledge, the only known mechanism for ceftiofur resistance in *Staphylococcus* is methicillin resistance (Park et al., 2013). It is reported that the *mecA* gene from S. sciuri was the precursor of the *mecA* gene found in clinical *S. aureus* currently related to infections in human being, and *S. sciuri* is the ancestral repository of *mecA* gene (Saraiva et al., 2021). An interesting finding here was the presence of sensitive isolates to ceftiofur but at the same time *mecA* positive, which will lead to neglect the spread of the *mecA* gene. It is worth noting that there were the same mutations in the unstable HR strains H0-2(HR*HR, unstable), C11-2 (HR*S), and sensitive strain D12-5. However, there were extra two mutations in the D12-4 (HR*HR, stable). We speculated that these two mutations might be related to ceftiofur heteroresistance. But further studies are very necessary to evaluate the mechanisms of ceftiofur heteroresistance and to verify that the point mutations are related ceftiofur heteroresistance.

The incidence of ceftiofur heteroresistance may be of concern if heteroresistance is a precursor to resistant strains (Band et al., 2021). If the selective pressure of continued exposure to antibiotics increases the possibility of the resistant isolates to the antibiotic, there may be more and more isolates resistant to ceftiofur occurring with a more widespread use of ceftiofur. Although the mechanism of ceftiofur heteroresistance on *Staphylococcus* is still unclear, the emergence of heteroresistance should be paid attention to.

5.6 Conclusion

In conclusion, our research is the first study to determine Staphylococcus strains displaying ceftiofur heteroresistance collected from milk. The mutations in the

mecA gene might be the primary mechanisms in the ceftiofur heteroresistance Staphylococcus. Our research underlies the significance and emergency of monitoring the presence of ceftiofur heteroresistance Staphylococcus in the susceptibility testing of Staphylococcus collected from milk. It is suggested that dairy cow mastitis inducing microorganisms should be concerned not only with regard to the antibiotic resistance but also about the antibiotic heteroresistance, thereby to reduce the risk of antibiotic resistance. The small sample size of Staphylococcus strains in our research limits the generalizability of the results. It is necessary to evaluate the frequency of ceftiofur heteroresistance in a larger size of Staphylococcus to validate these results.

5.7 Acknowledgments

This work was supported by the National Key R&D Program of China (No. 2022YFD1301004), Project of Risk Assessment on Raw Milk (GJFP20220304), the earmarked fund (CARS36), and the Agricultural Science and Technology Innovation Program (ASTIP-IAS12).

5.8 Supplemental Information

The study on the heteroresistance to cephalosporins of Staphylococcus in milk from mastitis suffering cows

Lei Dong, a,b,c Lu Meng, a,b Huimin Liu, a,b Nan Zheng, a,b Martine Schroyenc, Jiaqi Wang a,b#

[1] Institute of Crop Sciences (ICS), Chinese Academy of Agricultural Sciences (CAAS), Beijing 100081, China

[2] Hainan Yazhou Bay Seed Laboratory/National Nanfan Research Institute (Sanya), Chinese Academy of Agricultural Sciences, Sanya 572024, Hainan Province, China

[a] Key Laboratory of Quality & Safety Control for Milk and Dairy Products of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[b] Laboratory of Quality and Safety Risk Assessment for Dairy Products of Ministry of Agriculture and Rural Affairs, Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, P. R. China

[c] Precision Livestock and Nutrition Laboratory, Teaching and Research Centre (TERRA), Gembloux Agro-Bio Tech, University of Liège, Gembloux, Belgium

Contents

Table S1 Antibiotic susceptibility of 151 Stapphylococcus isolates																	
Area	Isolates name	Penicillin	Ampicillin	Amoxicillin/clavulanic acid	Oxacillin	Cephalothin	Ceftiofur	Erythromycin	Clindamycin	Gentamicin	Doxycycline	Florfenicol	Rifampin	Vancomycin	Ciprofloxacin	Sulfisoxazole	Trimethoprim/sulfamethoxazole
Qingdao	QDWY-2-1-2	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	0.5	≤0.25	1	≤0.25	1	≤0.25	8	≤0.12/2.4
	QDM-M-2-2-1-1	2	2	0.5/0.25	≤0.25	≤0.25	1	＞128	≤0.25	8	≤0.25	2	≤0.25	0.5	16	4	≤0.12/2.4
	QDM-M-3-2-5-1	1	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	8	≤0.25	8	0.5	8	≤0.25	≤0.5	16	4	≤0.12/2.4
	QDF-1-1-2	4	1	0.5/0.25	32	32	64	16	8	8	≤0.25	2	4	0.5	0.5	>1024	≤0.12/2.4
	QDM-M-3-3-5-1	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	2	≤0.25	0.5	≤0.25	32	≤0.12/2.4
	QDS-S-1-24	8	4	0.5/0.25	≤0.25	≤0.25	0.5	＞128	＞128	32	≤0.25	1	≤0.25	0.5	≤0.25	8	0.5/0.95
	QDM-S-4-17	8	8	0.5/0.25	≤0.25	≤0.25	0.5	＞128	＞128	32	≤0.25	1	≤0.25	0.5	1	8	0.5/0.95

	QDM-S-4-24	2	2	0.5/0.25	≤0.25	≤0.25	0.5	＞128	1	8	≤0.25	2	≤0.25	0.5	16	4	0.12/0.24
	QDM-S-8'-30	8	8	0.5/0.25	≤0.25	0.5	0.5	>128	>128	32	≤0.25	2	≤0.25	0.5	1	16	0.5/9.5
Xinjiang	d27	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	1	1	≤0.25	0.5	≤0.25	≤2	≤0.12/2.4
	d28	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	4	≤0.12/2.4
	d29	4	2	0.5/0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	≤0.25	1	8	≤0.12/2.4
	d30	≤0.25	≤0.25	≤0.25/0.12	1	0.5	8	32	≤0.25	32	≤0.25	2	≤0.25	1	≤0.25	1024	≤0.12/2.4
	d31	4	1/0.5	4	≤0.25	≤0.25	4	2	≤0.25	1	1	4	0.5	4	≤0.25	2	≤0.125/2.4
	d32	2	1	0.5/0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	0.5	4	≤0.12/2.4
	d33	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	0.5	≤0.25	0.5	≤0.25	256	≤0.12/2.4
	d34	1	0.5	≤0.25/0.1	8	16	2	1	16	4	≤0.2	1	1	0.5/0	0.5/0.	1024	≤0.12/2.4

			2							5			.25	25		
	103	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	≤2	≤0.12/2.4
	104	16	8	0.5/0.25	≤0.25	0.5	0.5	>128	>128	≤0.25	≤0.25	2	≤0.25	0.5	0.5	4	≤0.12/2.4
	105	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	4	≤0.12/2.4
	107	0.5	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	>128	>128	4	≤0.25	2	≤0.25	0.5	4	4	≤0.12/2.4
	113	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	4	≤0.12/2.4
	118	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	>128	>128	≤0.25	≤0.25	0.5	≤0.25	0.5	0.5	4	≤0.12/2.4
	120	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	≤2	≤0.12/2.4
	124	16	16	0.5/0.25	≤0.25	0.5	0.5	>128	>128	≤0.25	≤0.25	1	≤0.25	0.5	0.5	128	≤0.12/2.4
	J68	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	4	≤0.12/2.4
	J70	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	>128	>128	≤0.25	≤0.25	2	≤0.25	0.5	0.5	4	≤0.12/2.4
	J71	16	16	1/0.5	0.5	0.5	0.5	>128	>128	≤0.25	≤0.25	2	≤0.25	0.5	0.5	256	≤0.12/2.4
	J73	≤0.	≤0.2	≤0.25/0.1	≤0.	≤0.2	0.5	≤0.25	≤0.2	≤0.2	≤0.2	1	≤0.	0.5	0.5	4	≤0.12/2.4

		25	5	2	25	5			5	5	5		25				
	J74	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	4	≤0.12/2.4
	J76	≤0.25	≤0.25	≤0.25/0.12	1	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	8	≤0.12/2.4
	J82	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	0.5	4	≤0.12/2.4
	J83	16	16	0.5/0.25	≤0.25	0.5	0.5	> 128	> 128	≤0.25	≤0.25	2	≤0.25	0.5	0.5	1024	≤0.12/2.4
	J84	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	2	≤0.25	0.5	0.5	4	≤0.12/2.4
	J86	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	> 128	> 128	≤0.25	≤0.25	2	≤0.25	0.5	1	128	≤0.12/2.4
	J87	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	> 128	> 128	≤0.25	≤0.25	2	≤0.25	0.5	0.5	4	≤0.12/2.4
	J88	≤0.25	≤0.25	≤0.25/0.12	0.5	0.5	1	2	≤0.25	≤0.25	≤0.25	2	≤0.25	1	≤0.25	512	≤0.12/2.4
	J93	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	> 128	> 128	≤0.25	≤0.25	2	≤0.25	0.5	0.5	4	≤0.12/2.4
	J94	1	0.5	≤0.25/0.12	≤0.25	≤0.25	1	≤0.25	≤0.25	≤0.25	1	0.5	≤0.25	0.5	≤0.25	1024	≤0.12/2.4
	J96	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	2	≤0.25	0.5	0.5	4	≤0.12/2.4
Inn	G-229-	1	0.5	≤0.25/0.1	≤0.	≤0.2	≤0.	>	≤0.2	≤0.2	≤0.2	1	≤0.	0.5	≤0.25	16	≤0.12/2.4

er Mongolia	28-1			2	25	5	25	128	5	5	5		25				
	G-557-2-1	1	0.5	≤0.25/0.12	8	8	2	2	2	8	≤0.25	1	1	0.5	0.5	1024	≤0.12/2.4
	R-229-26-1	1	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	G-229-6-1	1	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	G-229-27-1	0.5	0.5	≤0.25/0.12	16	16	2	2	2	8	≤0.25	1	1	1	1	> 1024	≤0.12/2.4
	G-229-30-1	1	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	G-229-1-1	1	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	G-229-8-1	0.5	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	G-871-28-1	1	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	R-229-25-1	2	0.5	≤0.25/0.12	8	16	32	2	2	8	≤0.25	2	0.5	1	1	1024	≤0.12/2.4
	G-229-5-1	2	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	32	≤0.12/2.4
	18-tank-1	1	0.5	≤0.25/0.12	≤0.25	≤0.25	≤0.25	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	16-	128	32	0.5/0.25	0.5	≤0.2	0.5	2	16	≤0.2	≤0.2	2	≤0.	0.5	≤0.25	8	≤0.12/2.4

	1558-1					5				5	5		25				
	17-1745-2	128	64	1/0.5	0.5	≤0.25	2	> 128	128	≤0.25	≤0.25	2	≤0.25	0.5	≤0.25	8	≤0.12/2.4
	17-1509-1	8	4	0.5/0.25	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤2	≤0.12/2.4
	23-321-1	32	16	1/0.5	≤0.25	≤0.25	1	32	128	≤0.25	≤0.25	0.5	≤0.25	0.5	≤0.25	≤2	≤0.12/2.4
	18-tank-3	32	16	1/0.5	0.5	0.5	1	> 128	≤0.25	≤0.25	≤0.25	1	≤0.25	≤0.25	≤0.25	≤2	≤0.12/2.4
	16-1745-1	32	16	1/0.5	≤0.25	≤0.25	1	128	128	≤0.25	≤0.25	4	≤0.25	0.5	≤0.25	≤2	≤0.12/2.4
	21-7121-1	32	16	1/0.5	≤0.25	≤0.25	1	> 128	128	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	8	≤0.12/2.4
	20-tank-2	128	64	1/0.5	0.5	≤0.25	>128	≤0.25	≤0.25	≤0.25	≤0.25	2	≤0.25	0.5	≤0.25	4	≤0.25
	19-1745-1	64	32	1/0.5	≤0.25	≤0.25	1	> 128	128	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	≤2	≤0.12/2.4
	17-tank-1	64	16	1/0.5	0.5	≤0.25	5	> 128	≤0.25	≤0.25	≤0.25	2	≤0.25	0.5	≤0.25	8	≤0.12/2.4
	17-14F24-1	0.5	≤0.25	≤0.25/0.12	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	1	≤0.25	4	≤0.12/2.4
	15-1643-1	16	4	4/2	≤0.25	≤0.25	0.5	2	1	2	≤0.25	1	≤0.25	0.5	≤0.25	1024	≤0.12/2.4

	22-4958-2	8	8	1/0.5	0.5	≤0.25	1	>128	32	≤0.25	≤0.25	2	≤0.25	≤0.25	0.5	256	≤0.12/2.4
	22-1046-1	32	8	1/0.5	0.5	≤0.25	1	>128	128	≤0.25	≤0.25	1	≤0.25	≤0.25	≤0.25	8	≤0.12/2.4
	18-A190-1	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	≤0.25	>128	64	2	1	1	≤0.25	0.5	≤0.25	4	≤0.12/2.4
	23-190-1	32	8	1/0.5	0.5	≤0.25	1	>128	32	≤0.25	≤0.25	1	≤0.25	≤0.25	0.5	256	≤0.12/2.4
	17-1745-1	32	16	1/0.5	0.5	≤0.25	1	>128	128	≤0.25	≤0.25	2	≤0.25	≤0.25	≤0.25	8	≤0.12/2.4
	16-14F24-1	32	16	1/0.5	≤0.25	≤0.25	1	>128	>128	≤0.25	≤0.25	2	≤0.25	0.5	≤0.25	4	≤0.12/2.4
	23-JD-1	2	1	0.5/0.25	≤0.25	≤0.25	0.5	>128	128	≤0.25	≤0.25	2	≤0.25	0.5	≤0.25	128	≤0.12/2.4
	21-JD-2	2	1	0.5/0.25	16	16	16	4	4	8	≤0.25	2	1	2	0.5	>1024	≤0.12/2.4
	23-PF-4865-1	32	16	1/0.5	0.5	≤0.25	1	>128	128	≤0.25	≤0.25	2	≤0.25	≤0.25	≤0.25	256	≤0.12/2.4
	16-PF-1745-2	16	1	0.5/0.25	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	23-PF-4913-1	2	1	0.5/0.25	≤0.25	≤0.25	≤0.25	>128	≤0.25	≤0.25	≤0.25	1	≤0.25	≤0.25	≤0.25	32	≤0.12/2.4

	16-PF-A190-3	2	1	0.5/0.25	8	16	16	4	4	8	≤0.25	2	1	2	0.5	> 1024	≤0.12/2.4
	21-PF-0509-1	≤0.25	≤0.25	≤0.25/0.12	≤0.25	≤0.25	0.5	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	0.5	≤0.25	16	≤0.12/2.4
	23-PF-4965-3	≤0.25	≤0.25	≤0.25/0.12	≤0.25	0.5	≤0.25	16	8	4	≤0.25	2	2	0.5	0.5	1024	≤0.12/2.4
	16-PF-14F24-3	8	4	1/0.5	≤0.25	≤0.25	1	> 128	128	≤0.25	≤0.25	1	≤0.25	≤0.25	≤0.25	≤2	≤0.12/2.4
	23-PF-190-4	4	2	0.5/0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	1	≤0.25	≤0.25	≤0.25	≤2	≤0.12/2.4
	23PF-6218-2	1	≤0.25	≤0.25/0.12	≤0.25	≤0.25	≤0.25	16	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	0.5	≤0.25	≤2	≤0.12/2.4
Tianjin	H7-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	>1024	0.12/2.4
	M9-2	0.5	0.125	0.25/0.12	0.5	0.25	0.25	0.5	0.5								
	N9-5	0.125	0.125	0.25/0.12	0.5	0.5	0.25	8	0.25	0.25	0.25	4	0.125	0.5	0.125	32	0.12/2.4
	N1-6	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	4	0.25	0.5	0.125	1024	0.12/2.4
	N6-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	32	0.12/2.4

	C11-2	0.125	0.125	0.25/0.12	2	0.25	0.25	0.5	0.5	0.25	4	0.5	0.125	0.25	0.125	16	0.12/2.4
	G14-4	0.125	0.125	0.25/0.12	0.5	0.25	0.5	0.5	0.125	0.25	0.25	4	0.25	0.5	0.125	128	0.12/2.4
	G12-3	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	1	0.125	256	0.12/2.4
	M8-1	0.125	0.5	0.25/0.12	0.25	0.25	0.25	8	0.25	0.25	0.5	1	0.5	0.25	0.125	512	0.12/2.4
	M13-3	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.5	0.125	0.25	0.125	256	0.12/2.4
	H13-4	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	128	0.12/2.4
	H11-3	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	1	0.125	0.25	0.125	256	0.12/2.4
	C8-2	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.5	0.25	0.25	0.25	2	0.125	0.25	0.25	256	0.12/2.4
	N2-4	0.125	0.25	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	256	0.12/2.4
	P6-4	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	128	0.12/2.4
	M2-3	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	256	0.12/2.4
	C11-1	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	256	0.12/2.4

	H0-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	8	0.12/2.4
	H12-4	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	128	0.12/2.4
	N0-1	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	2	0.12/2.4
	D2-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	32	0.12/2.4
	C6-4	0.125	0.25	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	1	0.125	0.25	0.125	>1024	0.12/2.4
	P1-1	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	256	0.12/2.4
	D6-3	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	128	0.12/2.4
	C2-2	0.125	0.25	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	128	0.12/2.4
	Q12-4	0.125	0.25	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.25	0.25	0.125	>1024	0.12/2.4
	P11-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.5	0.25	0.25	0.25	2	0.125	0.25	0.125	256	0.12/2.4
	N1-4	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	256	0.12/2.4
	H3-5	8	16	1/0.5	128	16	8	0.25	0.25	0.25	0.5	0.5	0.25	0.25	0.125	32	0.12/2.4

	N8-5	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.5	0.125	0.25	4	2	0.125	0.5	0.125	1024	0.25/4.8
	N14-1	0.125	0.125	2/1	0.25	0.5	0.25	128	0.125	0.25	0.25	0.25	0.125	0.25	0.125	2	0.12/2.4
	Q3-3	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	64	0.12/2.4
	G3	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	1	0.125	0.25	0.125	>1024	0.12/2.4
	Q7-1	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	2	0.125	128	0.12/2.4
	D12-5	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.5	0.25	0.25	0.25	2	0.125	0.25	0.25	16	0.12/2.4
	D7-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	1	0.125	1	0.125	256	0.12/2.4
	D14-3	0.5	1	0.25/0.12	1	0.5	0.5	64	0.125	8	2	2	0.125	0.5	0.125	512	4/76
	N0-3	0.125	0.25	0.25/0.12	1	0.25	0.5	0.5	0.25	0.25	0.25	2	0.125	0.5	0.25	32	0.12/2.4
	M6-4	0.25	1	0.25/0.12	1	1	1	32	0.5	0.25	0.25	4	0.25	0.5	0.125	64	0.12/2.4
	C9-2	0.125	0.25	1/0.5	0.25	0.5	0.5	1	1	0.25	0.25	2	0.125	0.25	0.5	1024	0.5/9.5
	N2-2	4	8	8/4	64	4	8	0.5	0.25	0.25	2	1	0.125	0.25	0.125	128	0.12/2.4

	N14-2	0.125	0.125	2/1	0.25	0.25	0.5	2	0.125	0.25	0.25	0.25	0.25	0.25	0.125	4	0.25/8.4
	M6-6	0.125	0.125	0.25/0.12	0.5	0.25	0.25	>128	>64	0.25	>128	128	>64	0.25	16	>1024	>64/1216
	H11-1	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.25	0.5	0.125	128	0.12/2.4
	G7	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.25	0.5	0.125	64	0.12/2.4
	H9-2	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.25	0.125	0.25	0.25	2	0.25	0.5	0.125	32	0.12/2.4
	H8-3	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.25	0.125	0.25	0.25	2	0.25	0.5	0.125	256	0.12/2.4
	H1-1	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	1	0.125	128	0.12/2.4
	Q12-3	0.125	0.25	0.25/0.12	1	0.25	0.25	0.5	0.25	0.25	0.25	4	0.25	0.25	0.5	>1024	0.12/2.4
	M2-4	0.25	0.5	0.25/0.12	0.5	0.25	0.25	>128	>64	4	2	2	0.125	0.5	0.125	512	0.12/2.4
	M3-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	128	0.12/2.4
	M7	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	1	0.125	0.5	0.125	128	0.12/2.4
	H0-1	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	8	0.12/2.4

	M13	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.25	0.125	0.25	0.25	4	0.25	0.5	0.125	256	0.12/2.4
	H2-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	16	0.125	0.25	0.25	0.25	0.125	0.25	0.125	2	0.12/2.4
	H13-2	0.125	0.25	0.25/0.12	1	0.25	0.5	0.25	0.5	0.25	0.25	4	0.125	0.5	0.25	512	0.5/9.5
	P12-3	0.125	0.25	0.25/0.12	1	0.25	0.5	0.25	0.5	0.25	0.25	0.25	0.125	0.25	0.125	128	0.12/2.4
	P2-1	0.125	0.25	0.25/0.12	0.5	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.25	0.125	64	0.12/2.4
	G12-1	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	2	0.12/2.4
	N6-3	0.125	0.25	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	256	0.12/2.4
	G7-1	0.125	0.25	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.125	0.5	0.125	256	0.12/2.4
	H8-5	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	2	0.25	0.5	0.125	256	0.12/2.4
	C13	0.125	0.125	0.25/0.12	0.25	0.25	0.25	4	8	0.25	0.25	2	0.125	0.5	0.125	16	0.12/2.4
	P7-2	0.125	0.125	0.25/0.12	0.25	0.25	0.25	0.25	0.125	0.25	0.25	0.25	0.125	0.25	0.125	256	0.12/2.4
	H12-4	0.125	0.125	0.25/0.12	0.5	0.25	0.25	0.25	0.125	0.25	0.25	4	0.125	0.5	0.125	256	0.12/2.4

	D12-4	0.125	1	0.25/0.12	1	0.25	0.25	0.25	0.5	0.25	0.25	4	0.5	0.5	0.25	64	0.12/2.4
	M9-3	0.125	0.125	0.25/0.12	0.25	0.25	0.25	4	0.125	0.25	0.25	4	0.125	0.5	0.125	32	0.12/2.4
	C7-4	0.125	0.125	0.25/0.12	0.25	0.5	0.5	2	0.125	0.25	0.25	2	0.25	0.5	0.125	32	0.12/2.4
	P12-1	0.125	0.25	0.25/0.12	1	0.5	0.5	0.5	1	0.25	0.25	4	0.125	0.25	0.25	16	0.12/2.4
	G8-2	0.125	0.25	0.25/0.12	1	0.25	0.25	0.5	1	0.25	0.25	4	0.25	0.5	0.25	32	0.12/2.4

Table S2 Their multiple antibiotic resistance (MAR) index of 151 Staphylococcus isolates					
Isolates name	MAR index	Isolates name	MAR index	Isolates name	MAR index
QDWY-2-1-2	0	18-tank-1	0.13	D2-2	0
QDM-M-2-2-1-1	0.19	16-1558-1	0.13	C6-4	0.06
QDM-M-3-2-5-1	0.19	17-1745-2	0.25	P1-1	0
QDF-1-1-2	0.38	17-1509-1	0.06	D6-3	0
QDM-M-3-3-5-1	0	23-321-1	0.25	C2-2	0
QDS-S-1-24	0.25	18-tank-3	0.19	Q12-4	0.06
QDM-S-4-17	0.25	16-1745-1	0.25	P11-2	0
QDM-S-4-24	0.19	21-7121-1	0.25	N1-4	0
QDM-S-8'-30	0.25	20-tank-2	0.19	H3-5	0.25
d27	0	19-1745-1	0.25	N8-5	0.06
d28	0	17-tank-1	0.19	N14-1	0.13
d29	0.06	17-14F24-1	0	Q3-3	0
d30	0.25	15-1643-1	0.19	G3	0.06
d31	0.06	22-4958-2	0.25	Q7-1	0
d32	0.06	22-1046-1	0.25	D12-5	0
d33	0	18-A190-1	0.13	D7-2	0
d34	0.25	23-190-1	0.25	D14-3	0.25
103	0	17-1745-1	0.25	N0-3	0
104	0.19	16-14F24-1	0.25	M6-4	0.06
105	0	23-JD-1	0.19	C9-2	0.13
107	0.19	21-JD-2	0.25	N2-2	0.25
113	0	23-PF-4865-1	0.25	N14-2	0.06
118	0.13	16-PF-1745-2	0.06	M6-6	0.31
120	0	23-PF-4913-1	0.13	H11-1	0
124	0.19	16-PF-A190-3	0.25	G7	0
J68	0	21-PF-0509-1	0	H9-2	0
J70	0.13	23-PF-4965-3	0.19	H8-3	0

J71	0.25	16-PF-14F24-3	0.25	H1-1	0
J73	0	23-PF-190-4	0.06	Q12-3	0.06
J74	0	23PF-6218-2	0.06	M2-4	0.25
J76	0	H7-2	0.06	M3-2	0
J82	0	M9-2	0	M7	0
J83	0.25	N9-5	0.06	H0-1	0
J84	0	N1-6	0.06	M13	0
J86	0.13	N6-2	0	H2-2	0.06
J87	0.13	C11-2	0	H13-2	0.06
J88	0.06	G14-4	0	P12-3	0
J93	0.13	G12-3	0	甘 P2-1	0
J94	0.13	M8-1	0.19	甘 G12-1	0
J96	0	M13-3	0	N6-3	0
G-229-28-1	0.13	H13-4	0	G7-1	0
G-557-2-1	0.19	H11-3	0	H8-5	0
R-229-26-1	0.13	C8-2	0	C13	0.06
G-229-6-1	0.13	N2-4	0	P7-2	0
G-229-27-1	0.19	P6-4	0	H12-4	0
G-229-30-1	0.13	M2-3	0	D12-4	0.06
G-229-1-1	0.13	C11-1	0	M9-3	0
G-229-8-1	0.13	H0-2	0	C7-4	0
G-871-28-1	0.13	H12-4	0	P12-1	0
R-229-25-1	0.25	N0-1	0	G8-2	0
G-229-5-1	0.13				

Table S3 Genomic features of nine Staphylococcus spp. isolates

Feature	D12-4	H0-2	N0-3	C8-2	C11-2	Q12-3	H0-1	D12-5	C11-1
G+C content, %	32.6	32.49	32.62	32.55	32.6	36.91	36.88	32.61	36.88
Plasmids, no.	0	0	0	0	0	2	1	0	1
Chromosome	2684117	2678012	2790798	2667072	2639642	2368681	2331091	2657520	2330594
Genes, no.	2729	2901	2717	2770	2663	2601	2277	2680	2273
tRNA, copy no.	57	56	58	50	57	60	60	58	60
sRNA, copy no.	11	11	19	10	11	22	23	13	21

5.9 References

Andersson, D. I., Nicoloff, H., & Hjort, K. (2019). Mechanisms and clinical relevance of bacterial heteroresistance. Nature reviews. Microbiology, 17(8), 479–496.

Band, V. I., & Weiss, D. S. (2021). Heteroresistance to beta-lactam antibiotics may often be a stage in the progression to antibiotic resistance. PLoS biology, 19(7), e3001346.

Blasco, M. D., Esteve, C., & Alcaide, E. (2008). Multiresistant waterborne pathogens isolated from water reservoirs and cooling systems. Journal of applied microbiology, 105(2), 469–475.

Casaes Nunes, R. S., Pires de Souza, C., Pereira, K. S., Del Aguila, E. M., & Flosi Paschoalin, V. M. (2016). Identification and molecular phylogeny of coagulase-negative staphylococci isolates from Minas Frescal cheese in southeastern Brazil: Superantigenic toxin production and antibiotic resistance. Journal of dairy science, 99(4), 2641–2653.

Clinical and Laboratory Standards Institute [CLSI]. (2018). Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. CLSI supplement VET08. 4th edn. Wayne, PA: Clinical and Laboratory Standards Institute.

da Silva Cândido, T.J., da Silva, A.C., de Matos, L.G., da Silva do Nascimento, M., Camargo, C.H., Cobo Zanella, R., Mores Rall, V.L., & Cirone Silva, N.C. (2020). Enterotoxigenic potential and molecular typing of Staphylococcus sp. isolated from organic and conventional fresh minas cheese in the state of São Paulo, Brazil. International Dairy Journal, 102, 104605.

Donadu, M. G., Ferrari, M., Mazzarello, V., Zanetti, S., Kushkevych, I., Rittmann, S. K. R., Stájer, A., Baráth, Z., Szabó, D., Urbán, E., & Gajdács, M. (2022). No Correlation between Biofilm-Forming Capacity and Antibiotic Resistance in Environmental Staphylococcus spp.: In Vitro Results. Pathogens (Basel, Switzerland), 11(4), 471.

Dong, L., Meng, L., Liu, H., Wu, H., Hu, H., Zheng, N., Wang, J., & Schroyen, M. (2021). Effect of therapeutic administration of β-lactam antibiotics on the bacterial community and antibiotic resistance patterns in milk. Journal of dairy science, 104(6), 7018–7025.

El-Halfawy, O. M., & Valvano, M. A. (2015). Antimicrobial heteroresistance: an emerging field in need of clarity. Clinical microbiology reviews, 28(1), 191–207.

European Committee on Antimicrobial Susceptibility Testing. (2019). Breakpoint tables for interpretation of MICs and zone diameters, version 9.0.

Fluit A. C. (2012). Livestock-associated Staphylococcus aureus. Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 18(8), 735–744.

Hong, Y. K., Kim, H., & Ko, K. S. (2020). Two types of colistin heteroresistance in Acinetobacter baumannii isolates. Emerging microbes & infections, 9(1), 2114–2123.

Hung, K. H., Wang, M. C., Huang, A. H., Yan, J. J., & Wu, J. J. (2012). Heteroresistance to cephalosporins and penicillins in Acinetobacter baumannii. Journal of clinical microbiology, 50(3), 721–726.

Kelley, P. G., Gao, W., Ward, P. B., & Howden, B. P. (2011). Daptomycin non-susceptibility in vancomycin-intermediate Staphylococcus aureus (VISA) and

heterogeneous-VISA (hVISA): implications for therapy after vancomycin treatment failure. The Journal of antimicrobial chemotherapy, 66(5), 1057–1060.

Krumperman P. H. (1983). Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. Applied and environmental microbiology, 46(1), 165–170.

Leite, R. F., Gonçalves, J. L., Peti, A. P. F., Figueiró, F. S., Moraes, L. A. B., & Santos, M. V. (2018). Antimicrobial activity of crude extracts from actinomycetes against mastitis pathogens. Journal of dairy science, 101(11), 10116–10125.

Ma, W., Sun, J., Yang, S., & Zhang, L. (2016). Epidemiological and clinical features for cefepime heteroresistant Escherichia coli infections in Southwest China. European journal of clinical microbiology & infectious diseases: official publication of the European Society of Clinical Microbiology, 35(4), 571–578.

Milala, J., Piekarska-Radzik, L., Sójka, M., Klewicki, R., Matysiak, B., & Klewicka, E. (2021). Rosa spp. Extracts as a Factor That Limits the Growth of Staphylococcus spp. Bacteria, a Food Contaminant. Molecules (Basel, Switzerland), 26(15), 4590.

Morand, B., & Mühlemann, K. (2007). Heteroresistance to penicillin in Streptococcus pneumoniae. Proceedings of the National Academy of Sciences of the United States of America, 104(35), 14098–14103.

Nicoloff, H., Hjort, K., Levin, B. R., & Andersson, D. I. (2019). The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. Nature microbiology, 4(3), 504–514.

Odjadjare, E. E., Igbinosa, E. O., Mordi, R., Igere, B., Igeleke, C. L., & Okoh, A. I. (2012). Prevalence of multiple antibiotics resistant (MAR) Pseudomonas species in the final effluents of three municipal wastewater treatment facilities in South Africa. International journal of environmental research and public health, 9(6), 2092–2107.

Park, J., Friendship, R. M., Weese, J. S., Poljak, Z., & Dewey, C. E. (2013). An investigation of resistance to β-lactam antimicrobials among staphylococci isolated from pigs with exudative epidermitis. BMC veterinary research, 9, 211.

Pournaras, S., Ikonomidis, A., Markogiannakis, A., Maniatis, A. N., & Tsakris, A. (2005). Heteroresistance to carbapenems in Acinetobacter baumannii. The Journal of antimicrobial chemotherapy, 55(6), 1055–1056.

Saini, V., McClure, J. T., Léger, D., Keefe, G. P., Scholl, D. T., Morck, D. W., & Barkema, H. W. (2012). Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. Journal of dairy science, 95(8), 4319–4332.

Saraiva, M. M. S., de Leon, C. M. C. G., Silva, N. M. V. D., Raso, T. F., Serafini, P. P., Givisiez, P. E. N., Gebreyes, W. A., & Oliveira, C. J. B. (2021). Staphylococcus sciuri as a Reservoir of mecA to Staphylococcus aureus in Non-Migratory Seabirds from a Remote Oceanic Island. Microbial drug resistance (Larchmont, N.Y.), 27(4), 553–561.

Saravolatz, S. N., Martin, H., Pawlak, J., Johnson, L. B., & Saravolatz, L. D. (2014). Ceftaroline-heteroresistant Staphylococcus aureus. Antimicrobial agents and chemotherapy, 58(6), 3133–3136. https://doi.org/10.1128/AAC.02685-13

Søgaard P. (1985). Population analysis of susceptibility to cefotaxime in Enterobacteriaceae. Acta pathologica, microbiologica, et immunologica Scandinavica. Section B, Microbiology, 93(5), 365–369.

Tan, K., Nguyen, J., Nguyen, K., Huse, H. K., Nieberg, P. H., & Wong-Beringer, A. (2020). Prevalence of the carbapenem-heteroresistant phenotype among ESBL-producing Escherichia coli and Klebsiella pneumoniae clinical isolates. The Journal of antimicrobial chemotherapy, 75(6), 1506–1512.

Tegegne, H. A., Koláčková, I., Florianová, M., Gelbíčová, T., Madec, J. Y., Haenni, M., & Karpíšková, R. (2021). Detection and molecular characterisation of methicillin-resistant Staphylococcus aureus isolated from raw meat in the retail market. Journal of global antimicrobial resistance, 26, 233–238.

WHO. Critically Important Antimicrobials for Human Medicine, Fifth Revision. 2017. Available at: http://who.int/foodsafety/publications/antimicrobials-fifth/en/, accessed January 2, 2019.

Chapter 6

General discussion, conclusions and perspective

6.1 Evaluating Cephalosporin Usage in Mastitis Management: A Comprehensive Analysis

In this study, we explored the effect of cephalosporins on the microbiota and ARGs in milk and feces of dairy cows with mastitis. Cephalosporins are a class of β-lactam antibiotics that are commonly used to treat various infectious diseases by disrupting the peptidoglycan layer synthesis in the cell wall of bacteria (Magdaleno et al., 2015). Cephalosporins account for 50-70% of the total antibiotics used by humans. In Europe, cephalosporins have been considered as the second most prescribed antibiotic class until 2012 (Estrada et al., 2012). On dairy farms in the U.S., intramammary ceftiofur, a 3rd generation cephalosporin, is the most commonly used antibiotic for the treatment of mastitis. Ceftiofur was the only antibiotic used by all 40 large dairy farms included in a recent study, accounting for about half of all antibiotic use in dairy cows (de Campos et al., 2021). The WHO classifies ceftiofur as a "highest priority, critical important antibiotic" (Anonymous et al., 2019). In the Netherlands, the use of this critical important antibiotic is only allowed after diagnostics and susceptibility tests show that there are no alternatives (Koops et al., 2018).

Mastitis can be categorized based on various factors, including its duration, symptoms, and the responsible pathogenic agent. Inadequate management practices in dairy farming have also contributed to the increased incidence of mastitis. Such factors include high stocking density, subpar hygiene and sanitation, such as inadequate drainage systems and accumulation of dung, as well as the presence of flies and peri-parturient diseases (Bari et al., 2022). On many dairy farms, clinical mastitis is treated symptomatically without knowing the cause, but many of these treatments are unnecessary (Ruegg et al., 2019). On most farms, at least 85% of clinical mastitis cases are not severe when detected, so, immediate antibiotic treatment is not required (Oliveira et al., 2013). So, the timing of antibiotic administration is a crucial concern. Are farmers initiating antibiotic treatment promptly upon detecting mastitis, or is there a tendency to treat too early or too late? Early treatment may curb the progression of the infection, but it also raises concerns about antibiotic resistance development and the potential overuse of antibiotics. The U.S. Food and Drug Administration (FDA)-approved treatment with intramammary ceftiofur includes a flexible duration of 2 to 8 days. Although most veterinarians treat until clinical signs resolve (approximately 5 days), the benefits of longer treatment are outweighed by the cost (Oliveira et al., 2014; Pinzon-Sacnchez et al., 2011).

Lima et al., (2018) conducted the experiment to compared the microbiota from healthy and clinical mastitis cows. The milk samples were divided into four groups based on their health status: healthy, *E. coli*-mastitis, *Klebsiella* spp.-mastitis, and *Streptococcus* spp.-mastitis. Notably, the shared families among these groups exhibited a consistent mean relative abundance exceeding 85%, regardless of the milk-health-status. Taxonomic data at the family level showed that sequences from mastitis milk samples cultured positive for *E. coli* and *Klebsiella* spp. groups were predominantly affiliated with *Enterobacteriaceae*

(family), while for *Streptococcus* spp. group were dominated by *Streptococcacea* (family), followed by *Pseudomonadaceae* (family) and *Enterococcaceae* (family).

In the context of our experimental farm, the decision on the duration of antibiotic treatment for mastitis is guided by daily monitoring of SCC in raw milk. Elevated SCC can be indicative of mastitis, and this practice is used to assess the severity of the infection. In such cases, antibiotics are administered for a specific duration to address the infection. An easy method to reduce antibiotic use is to reduce the duration of intramammary therapy from 5 days to 3 days. Schukken et al. reported a 38% cure rate for the bacteria in untreated cows and a 73% cure rate in treated animals after a 5-day intramammary treatment with ceftiofur (Schukken et al., 2011). This treatment led to a significant increase in cure rate, especially in *E. coli*-infected animals. However, Ganda et al. showed no significant difference in cure rate between treated and untreated animals (Ganda et al., 2016). Consequently, there is evidence suggesting that we could reduce antibiotic use in a cattle herd without significantly compromising their health.

The great problem with the uncontrolled use of antibiotics lies clearly in the emergence of bacterial resistance. In the first experiment, the cephalosporin treatment did not have a significant effect on the relative abundance of *Staphylococcus* in milk. However, when analyzing the *Staphylococcus* isolated from the milk samples, they were almost all completely sensitive to ceftiofur (68/70, 97%) (data not shown in the experiment 1). If the *Staphylococcus* isolated from milk samples are truly sensitive to ceftiofur, the cephalosporin treatment should theoretically significantly reduce the relative abundance of *Staphylococcus*. Therefore, we hypothesized that *Staphylococcus* may have developed antibiotic heteroresistance (HR) due to the use of cephalosporins. Despite the clinical importance of cephalosporin-resistant Staphylococcus species, there is currently limited research available on this specific topic. This scarcity of studies means that our understanding of the resistance mechanisms employed by these bacteria is not as comprehensive as it is for other well-studied antibiotic-resistant strains. In experiment 1, we isolated four strains of *E. coli* from the raw milk samples (all present in samples taken during the medication period). Among them, one strain was found to be resistant to ceftiofur (the data was not shown due to the small sample size). Based on our observations, we speculate that mastitis in the dairy cows on this farm is mostly caused by *Staphylococcus* spp. rather than *E. coli*. However, the emergence of ceftiofur-resistant *E. coli* should be a matter of concern for us.

In recent studies, it is seen that resistance to β-lactam antibiotics, particularly to 3rd generation cephalosporins, is on the rise in commensal *Enterobacteriaceae* isolates from U.S. dairy cows (Gelalcha et al., 2022). Resistance to 3rd generation cephalosporins is mainly mediated by the production of extended-spectrum β-lactamases (ESBLs), which can destroy β-lactam rings of these 3rd generation cephalosporins (Rawat et al., 2010). Resistance to cephalosporins is widespread in human and animal *Enterobacterales* worldwide and is mediated mainly by two

classes of inactivating enzymes: extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC β-lactamases (AmpCs) (Harris et al., 2015). When examining different studies performed in the Netherlands, the ESBL/AmpC gene profile distribution of *E. coli* from livestock and food-associated species was found to differ from that of *E. coli* from humans (Dorado-García et al., 2018). Also in the UK, whole genome sequencing revealed that *E. coli* populations, including those with ESBL and AmpC genes, in livestock and retail meat were different compared to *E. coli* populations isolated from human blood samples taken after an *E. coli* infection (Ludden et al., 2019). To examine patterns from diverse countries and sources can offer further insights and enable comparisons about the patterns and dissemination of antimicrobial resistance. Hayer et al. reviewed that the percentage of swine possessing *E. coli* resistance for 3rd generation cephalosporins varied by country, region and the source of isolate sampling (healthy or diseased animals) (Hayer et al., 2022). In general, the frequencies of 3rd generation cephalosporin resistance were lower in isolates from Australia, European and North American countries. Not surprisingly, some of these countries have implemented policies restricting the use of antibiotics, including cephalosporins, in animal production (Agerso et al., 2013; Abraham et al., 2015; Callens et al., 2018). Chantziaras et al. found a high correlation between antibiotic use and resistance using country-level data from seven European countries (Chantziaras et al., 2014). In Asian countries, the proportion of 3rd generation cephalosporin-resistant *E. coli* isolates collected from pigs was very high. This is not surprisingly since the rate of antibiotic use in animal production is higher in Asian countries than in any other region in the world. Within the *Enterobacteriaceae* family, *E. coli* and *Klebsiella* are commonly identified bacteria that harbor ESBL-encoding genes, including $bla_{CTX\text{-}M}$ (cefotaxime-hydrolyzing β-lactamase), bla_{SHV} (sulfhydryl reagent variable (SHV) enzymes), and bla_{TEM} (TEM enzymes) (Collis et al., 2019). The parental types of SHV and TEM are narrow-spectrum β-lactamases, which are mutated to generate their respective ESBL variants. Amino acid substitutions or mutations in the genes responsible for these enzymes have the ability to enhance hydrolytic activity or improve substrate specificity (Castanheira et al., 2021). Consequently, there is an increasing number of identified variants in TEM and SHV families, most of which have emerged through stepwise mutations (Castanheira et al., 2021).

6.2 Cephalosporins resistance in animals and humans

The 3rd generation cephalosporin resistance is now a widespread and serious problem. In 2020, the 3rd generation cephalosporin resistance rates of invasive *Klebsiella pneumoniae* isolates were 50% or more in 44% of the countries reporting data to the European Antimicrobial Resistance Surveillance Network (EARS-Net), mainly in countries of Southern and Eastern Europe, such as Bulgaria (79.1%), Greece (74.5%), and Romania (67.9%) (WHO, 2021). It is reported that the use of ceftriaxone or ceftiofur increased fecal extended-spectrum cephalosporin (3rd and 4th generation) resistant *Enterobacterales* (ESCR-E) prevalence in hospitalized dogs during treatment and this persisted for 4 weeks

after discharge (Salgado-Caxito et al., 2021). It is also reported that the use of extended-spectrum cephalosporin (ESC) antibiotics in combination with hospitalization for more than 6 days increased the number of multidrug-resistant *E. coli* in the feces of the hospitalized patients (Gibson et al., 2011). A study by Sheedy et al. demonstrated a temporal association between antibiotic treatment and ceftiofur-resistant *Enterobacteriaceae* in early lactating dairy cows. Conversely, in untreated cows, the prevalence of the resistant Enterobacteriaceae phenotype was notably low, whereas cows treated systemically with antibiotics experienced rapid emergence of the AMR phenotype, peaking during treatment (Sheedy et al., 2021). ESC-resistant *Salmonella* has also been isolated from food-producing animals and their products in some European countries (Hasman et al., 2005; Rodriguez et al., 2009). It is reported that over the past decade these isolates are also particularly prevalent in chickens in Japan (Bueno et al., 2018).

Although AMR research and policy work have focused on the medical and animal husbandry, the wider environment as a conduit between human and animal hosts, with increasing evidence for the presence of AMR in watersheds and croplands, is receiving renewed attention (He et al., 2020; Rahman et al., 2021).

Consumption trends indicate an increasing preference for plant-based diets, which are often eaten with minimal preparation or heat treatment, and thus food crops may pose an important AMR hazard to consumers (Sivapalasingam et al., 2004). A growing number of observational investigations in the fields and markets report the presence of resistant microorganisms on food crops (Brunn et al., 2022a). Using a meta-analysis approach and providing a baseline from the existing literature, Brunn et al. found that 3.75% of food crops globally under investigation for AMR were contaminated with 3rd generation cephalosporin resistant *Enterobacteriaceae* (Brunn et al., 2022b). To date, food safety authorities have focused on foods of animal origin to characterize the AMR hazards related to veterinary use of antibiotics (Van Boeckel et al., 2019), but Brunn et al. consider plant-based foods to be an overlooked hazard of consumer and occupational AMR exposure (Brunn et al., 2022b).

6.3 Effects of cephalosporins on animal microbiota and resistance genes

The global debate on the public health implications of the use of antibiotics in animal production has been intense since recent decades. The reasons for this are not limited to the emergence and spread of zoonotic resistant pathogens (Bueno et al., 2018), but also include the risks related to the transmission of resistance genes to humans through the consumption of foods, or through some direct and indirect contact with environmental sources that are contaminated by waste from agricultural systems following the heavy use of antibiotics (Costa et al., 2017; Xiong et al., 2018). *Ruminococcus* are often associated with gut health through the production of short-chain fatty acids, which play an important role in reducing colonization by many opportunistic pathogens (Yu et al., 2018). A reduction of

Ruminococcus therefore is considered not opportune. In our study (chapter 4), *Ruminococcus* dominated the fecal samples, but the ceftiofur treatment had no significant reduction effect on the relative abundance of *Ruminococcus*. We did however observe a significant reduction of *Moraxellaceae* in feces after antibiotic treatment (chapter 4). *Moraxellaceae* is widely believed a pathogenic bacterium because it can cause human respiratory diseases such as asthma (Liu et al., 2019). *Moraxellaceae* has been detected in various medias such as milk, environmental samples, and bedding samples (Wu et al., 2020). However, we did not detect *Moraxellaceae* in our milk samples. *Moraxellaceae* is known for its ability to survive and reproduce at low temperatures, and can secrete protease and lipase, which can lead to gelatinization and odor of milk protein, and reduce the quality of milk (Wu et al., 2019). We also observed the relative abundance of *Clostridia*, *Clostridiales*, and *Clostridium*_IV in the feces decreased significantly with the cephalosporin treatment, some *Clostridia* are associated with pathogenic processes, even implicated in severe diseases such as infant botulism in preterm neonates (Grenda et al., 2022). In the milk, the treatment of cephalosporin decreased the relative abundance of *Enterobactor*, *Curvibacter*. With this in mind, the reduction of these bacteria would be favorable. This seems to favor the administration of ceftiofur in our experiments, but there needs to be a balance point with antibiotic use.

With regard to the resistance genes (ARGs), Alali et al. used fecal samples examined before by Lowrance et al. and they saw that there was an increase in the number of bla_{CMY} genes after the ceftiofur treatment (day 6) in the fecal microbiota compared with those present in the control cattle (Lowrance et al., 2007). On day 14, the gene copy numbers recovered to baseline levels (Alali et al., 2009). These results were also supported by a recently published microbiome study by Weinroth et al., which showed there were no significant differences in the β-lactamase resistant genes in the fecal microbiome collected before (day 0) and after (day 26) ceftiofur treatment in the obtained beef cattle (Weinroth et al., 2018). Therefore, the potential public health risk associated with the selection of ESBL-resistant microorganisms in cattle may depend largely on the time between the antibiotic treatment and slaughter. These studies support our results. In our study, we also observed a significant increase of the relative abundance of β-lactamase resistant genes in milk (bla_{TEM}) and feces (bla_{TEM} and *cfx*A) at the period of withdrawal (Chapter 3 & 4). The relative abundance of these two kinds of genes both decreased at the period of recovery (day 9 to day 15), but neither decreased to the level of day 0. Therefore, the long-term (>15 days) effect of the cephalosporin treatment on the fecal microbiota and resistome are worthy of further investigation.

Besides controlling the ARGs, it is also important to develop the appropriate management to control the transfer of ARGs. Levent et al. identified Inc class plasmids, which have been proven to transfer ESBL and AmpC resistance elements between *Salmonella* and *E. coli* (Levent et al., 2022). The IncA/C plasmid is one of the main plasmids carrying bla_{CMY-2}, while the $bla_{CTX-M-32}$ gene

was previously found in the IncN plasmid of beef cattle-derived *E. coli* (Call et al., 2010; Cottell et al., 2013). Under the selective pressure of ceftiofur, it was demonstrated that the Inc A/C plasmid displayed a high degree of stability (Subbiah et al., 2011). The proliferation of resistant endogenous *Enterobacteriaceae* after ceftiofur treatment may lead to refractory or even untreatable infections in the future. Furthermore, the gut microbiota may serve as a repository for these resistance genes (Penders et al., 2013). Conjugal transfer of plasmids carrying antibiotic resistance genes has been shown to occur frequently among *Enterobacteriaceae* in diverse environments such as milk, meat, and feces. This transfer can arise even in the absence of antibiotic pressure (Warnes et al., 2012). Moreover, studies have indicated that plasmids harboring bla genes can readily transfer from invasive Enterobacteriaceae to those residing in the gut of animals and humans (Goren et al., 2010). In our study, we did not find plasmids with the bla gene. However, we did detect antibiotic resistance genes coding for tetracycline resistance (*tet*(W) and *tet*(Q)) in the fecal samples while none of the cattle received tetracyclines during the study. This may be because the antibiotic use can provide selective pressure to maintain other unassociated resistance genes by linking to mobile genetic elements (MGEs), such as a plasmid (Enne et al., 2004). It has been reported that MGEs promote the mobilization and spread of ARGs in bacteria. Plasmids play an important role in the accumulation and transfer of ARGs, and are involved in the acquisition of resistance to most antibiotic classes including β-lactams (Shintani et al., 2015). Pig manure can also be a reservoir for the *bla*TEM genes, which are often embedded in IncN plasmids (Binh et al., 2008). IncN plasimids are also related to *bla*CTX-M genes, which have been observed in bacteria from pigs, farmers and manure (Moodley et al., 2009). Plasmid-mediated horizontal transfer of *bla*TEM genes has been reported between poultry and humans with high conjugative mobility (Singh et al., 2018). Our research allows for a visual assessment of the impact of cephalosporin usage in dairy cows with mastitis, specifically on the microbiota and ARGs of raw milk and feces. Of particular interest is the influence on raw milk, as there has been limited prior investigation into the effects of antibiotic usage on the microbial community and ARGs within raw milk. So, the resistant bacteria and resistance genes in the feces can also be seen as a serious problem because they may transfer among cattle herds and result in antibiotic treatment failure. Therefore, the appropriate use of antibiotics in dairy cattle is an important process to avoid the spread of ARBs and ARGs.

6.4 Bacterial heteroresistance is a problem that cannot be ignored

HR is characterized by the ability of a preexisting subpopulation of resistant cells that have rapid replication when exposed to specific antibiotics, while the majority of the susceptible cell population is killed (Band et al., 2019). HR differs from other forms of subpopulation-mediated resistance, such as persistent

resistance, in which a small number of transiently quiescent or very slow-growing bacterial subpopulations exhibit increased resistance to multiple antibiotics (Brauner et al., 2016). Persistence is thought to lead to the recurrence of infection after cessation of antibiotic therapy, but not to failure of acute therapy. HR is also distinct from tolerance, in which the entire bacterial population is able to survive temporary exposure to high concentrations of antibiotics, even in the absence of preexisting resistant cells prior to antibiotic exposure (Brauner et al., 2016).

The phenomenon of HR is very common and is considered to be a precursor stage, which may lead to the emergence of ARB (Falagas et al., 2008). Furthermore, HR is considered a natural evolutionary strategy for antimicrobial resistance, as it provides bacteria with the opportunity to explore growth in the presence of antibiotics before the acquisition of resistance by the major microbial populations (Morand et al., 2007). The HR phenomenon has emerged among clinical pathogens such as *Helicobacter pylori* (Kouhsari et al., 2022), *Pseudomonas aeruginosa* (Lu et al., 2022), *Staphylococcus aureus* (Bai et al., 2019), *Mycobacterium tuberculosis* (Werngren et al., 2021), *Acinetobacter baumannii* (Jo et al., 2021), *Streptococcus pneumoniae* (Lohsen et al., 2023), and *Klebsiella pneumoniae* (Cheong et al., 2019).

Currently, research on the mechanism of bacterial HR mainly concentrates on point mutations. Bacteria can generate subpopulations at a high fitness cost through point mutations under the selective pressure of antibiotic exposure or through compensatory secondary mutations in the absence of antibiotics (Kouhsari et al., 2022). In the case of HR, antibiotic susceptibility testing of pure clones would result in the detection of a fully sensitive or fully resistant phenotype, depending on which of the two populations (resistant or sensitive) the purified clones were derived from (EI-Halfawy et al., 2015). Genomic mutations often result in stable HR, in which the resistant phenotype remains stable and does not quickly revert to susceptibility even in the absence of antibiotic pressure. However, genomic mutations may also induce unstable HR, a very common kind of HR. This happens when the mutations are inherently unstable and gene tandem amplification or the resistance mutations confer a high adaptation cost, which drives the resistant subpopulation to regain susceptibility (Li et al., 2022). The stable HR can be attributed to low fitness-cost mutations that accumulated and evolved over many generations through selection processes. The cost of resistance must be compensated by reducing the growth of antibiotic-induced sensitive strains (Wang et al., 2021). The study by Kuang et al., identified that in *E. coli* showing colistin-HR, sampled from swine in China, mutations in PmrB and/or PhoQ were observed to be the major mechanisms of colistin-HR. The authors also found the presence of the R93P mutation within the PmrB HAMP domain resulted in its continuous activation, leading to the overexpression of *pmrB*, *pmrA*, *pmrC*, and *pmrHFIJKLM*, which is related to the formation of stable resistant subpopulations in colistin-HR *E. coli* (Kuang et al., 2020). Wang et al., found that HR observed in pure cyanobacterial isolates resulted in the frequent formation of spontaneous subpopulations of sensitive and resistant cells, and this when grown

without antibiotics, due to the reversibility of the resistance phenotype that attributed to an unstable genetic mechanism. Their results showed that more than 97% of HR cases observed in environmental isolates selected from PAP were unstable (Wang et al., 2021). A recent study exhibited that HR caused by the amplification of ARG is the most common type of HR in Gram-negative bacteria (Hjort et al., 2016). It was reported among 766 bacteria-antibiotic combinations tested, 27.4% showed HR, 88% of which showed unstable HR related to tandem gene amplification of known ARG (Nicoloff et al., 2019). Jayol et al., (Jayol et al., 2015) and Lee et al., (Lee et al., 2015) proposed two potential genetic explanations for HR to colistin, both of which involve stable genetic mutations. In a recent report, Band et al., highlighted the problem of unstable colistin heteroresistant clinical *Enterobacter cloacae* isolates that were misclassified as sensitive and resulted in treatment failure in a mouse model (Band et al., 2016). The antibiotic-resistant subpopulation appeared to be genetically identical to the sensitive cell population, but with increased transcript levels of the *arn* operon and *pmrC*, resulting in the increase of resistance. Other studies have shown that the unstable tobramycin resistance in *Acinetobacter baumannii* arises from extensive RecA-dependent amplification of the *aadB* gene encoding the aminoglycoside adenosyltransferase (Anderson et al., 2018).

Another mechanism leading to HR is the hyperproduction of efflux pumps and reduced expression of porins (Zheng et al., 2018; Chen et al., 2017). Zheng et al., found the overexpression of the periplasmic adapter MacAB in eravacycline HR in clinical *K. pneumoniae* isolates. The significant MacA expression levels detectedd in 12 eravacycline-heteroresistant bacteria indicated a potentially crucial role of the MacAB-TolC multidrug efflux pump in eravacycline HR within *K. pneumoniae*, as does OqxAB (Zheng et al., 2018). Chen et al. discovered that elevated levels of the RamA transcriptional factor resulted in the upregulation of both the AcrAB-TolC and OqxAB efflux pump systems. This cascade finally culminated in HR to tigecycline within *Salmonella enterica* (Chen et al., 2017). Similarly, it was reported that the reduction in OprD porin expression and the overexpression of efflux systems contribute to HR against carbapenems and imipenem in *Pseudomonas aeruginosa*. (Ikonomidis et al., 2008; Xu et al., 2020).

To survive treatment, the HR phenotype must be at least partially transmitted to daughter cells. For phenotypic HR without any genetic basis, it is unclear how this is achieved. Epigenetics may explain the maintenance of HR phenotypes over multiple generations. In fact, epigenetic inheritance of phenotypic traits has been shown to occur across generations (Ram et al., 2013; Sorg et al., 2015). However, this unstable phenotype is not maintained indefinitely, and the populations again accumulate antibiotic-sensitive cells and will revert to their initial phenotypic variation without antibiotic. When initiating antibiotic therapy, it is assumed that a single compound that is effective for all populations can easily eradicate the causative infectious agent. If the immune system fails to eradicate heteroresistant bacteria, the heteroresistant bacteria can readily survive antibiotic treatment and

prevent clearance of the infection. For example, the *Klebsiella pneumoniae* showed HR in vitro but cause therapy failure in vivo models (Band et al., 2016). Therefore, diagnostics should not only assess resistance but also consider population heterogeneity in heteroresistant forms. Currently, the most reliable method for assessing HR is performing the PAP assay. In the PAP assay, growth is measured at different antibiotic concentrations and compared to growth without antibiotic. A gradual decrease in growth rather than a single-step response indicates the presence of subpopulations with higher MIC values, namely heteroresistant subpopulation (Andersson et al., 2019). Unfortunately, PAP assay is very labor-intensive and thus not feasible to implement in clinical practice. There is currently no reliable, less labor-intensive method to detect HR (Andersson et al., 2019).

Until now, there are almost no reports on HR to ceftiofur in *Staphylococcus spp*. in food. As far as we know, our study is the first to investigate HR to ceftiofur in *Staphylococcus spp*. in milk. The *Staphylococcus* strains investigated in some previous reports were collected from people (Ma et al., 2016). It is also important and necessary to monitor the presence of HR when performing diagnostic susceptibility testing of *Staphylococcus* isolates in animals. Our study can draw attention to the use of ceftiofur as a treatment for mastitis in dairy cows and guide the appropriate use of antibiotics in animal husbandry. In our study, there were 15 isolates showing the possibility of inducible resistance to CEF using the disc diffusion method. Only three *Staphylococcus* strains that were determined to be sensitive to ceftiofur were subsequently identified as ceftiofur heteroresistant by the PAP method, as mentioned before, the most reliable method for determining HR. In the three HR strains, we found that only one strain was stable but the other two subpopulations were unstable and transient, and the MIC reverted to a lower level. Recently, studies have shown that HR often occurs in clinical isolates and that the majority of these strains are heteroresistant to more than one antibiotic (Nicoloff et al., 2019). Against this background, a combination of different antibiotics can be used to eradicate bacterial infections so that no subpopulation becomes resistant to all the antibiotics administered. This has proven to be an effective strategy (Band et al., 2019).

6.5 General conclusion and perspectives

The objective of this PhD research was to investigate the effect of 2 commonly used cephalosporins on the microbiota and the antibiotic resistance genes in the milk and feces of dairy cows, and to research the presence of heteroresistance *Staphylococcus* to ceftiofur in raw milk. We found that the treatment with cephalosporins lead to a change in the milk microbiota and an increase of the presence of the β-lactamase resistance gene in the milk at the time of withdrawal period. The cephalosporin treatment decreased the microbial diversity and richness at the medication period, and increased the relative abundance of two β-lactamase resistance genes at the withdrawal period. We also found three strains that exhibited heteroresistant phenotypes by the PAP method. One of these heteroresistant strains was stable, and there were two extra mutations in the

heteroresistant stable isolate, which might have resulted in the formation of a stable resistant subpopulation in heteroresistant *Staphylococcus*. These findings enhance concerns about the emergence of ceftiofur-heteroresistant *Staphylococcus* isolates and the application of ceftiofur as therapy for the treatment for mastitis in dairy cows.

The study of antibiotic resistance development and resistance mechanisms must be a mandatory requirement in the early stages of drug development. Knowledge of how and when resistance occurs, and potential synergies with antibiotic combinations, will also promote the development of dosing regimens that can help minimize resistance to current and new antibiotics, enabling these drugs work best. This is very important in the short term, as new drugs are unlikely to enter widespread clinical practice in the near future. The challenge now facing the field is to make full use of existing technology, information and expertise to ensure that the impact of resistance is fully taken into account in the urgent development of next-generation antibiotics. Newly developed antibiotics should also facilitate therapeutics that completely eradicate infectious pathogens in vivo, including subpopulations that are refractory to conventional antibiotic therapy.

To enhance our understanding of heteroresistance mechanisms, future studies should focus on several key areas. Firstly, comprehensive investigations can explore the specific genetic and molecular mechanisms that underlie antibiotic heteroresistance in pathogens. This could involve whole-genome sequencing and metagenomic analyses to identify genetic determinants and resistance genes associated with heteroresistance. Secondly, to identify specific bacterial strains or resistance mechanisms that are of particular concern due to their ability to maintain high fitness while carrying ARGs. Such strains may be more challenging to control and may require unique strategies. Additionally, research focused on the temporal dynamics of heteroresistance development can reveal how and when heteroresistance occurs during treatment. By exploring these avenues, we can develop a more profound understanding of heteroresistance and potentially identify novel strategies for its mitigation in antibiotic treatment.

6.6 References

Abraham, S., Jordan, D., Wong, H. S., Johnson, J. R., Toleman, M. A., Wakeham, D. L., Gordon, D. M., Turnidge, J. D., Mollinger, J. L., Gibson, J. S., & Trott, D. J. (2015). First detection of extended-spectrum cephalosporin- and fluoroquinolone-resistant Escherichia coli in Australian food-producing animals. Journal of global antimicrobial resistance, 3(4), 273–277.

Agersø, Y., & Aarestrup, F. M. (2013). Voluntary ban on cephalosporin use in Danish pig production has effectively reduced extended-spectrum cephalosporinase-producing Escherichia coli in slaughter pigs. The Journal of antimicrobial chemotherapy, 68(3), 569–572.

Alali, W. Q., Scott, H. M., Norby, B., Gebreyes, W., & Loneragan, G. H. (2009). Quantification of the bla (CMY-2) in feces from beef feedlot cattle administered three different doses of ceftiofur in a longitudinal controlled field trial. Foodborne pathogens and disease, 6(8), 917–924.

Andersson, D. I., Nicoloff, H., & Hjort, K. (2019). Mechanisms and clinical relevance of bacterial heteroresistance. Nature reviews. Microbiology, 17(8), 479–496.

Anderson, S. E., Sherman, E. X., Weiss, D. S., & Rather, P. N. (2018). Aminoglycoside Heteroresistance in Acinetobacter baumannii AB5075. mSphere, 3(4), e00271-18.

Anonymous. (2019). Critically Important Antimcrobials for Human Health, 6th Revision; WHO: Geneva, Switzerland.

Bai, B., Lin, Z., Pu, Z., Xu, G., Zhang, F., Chen, Z., Sun, X., Zheng, J., Li, P., Deng, Q., & Yu, Z. (2019). In vitro Activity and Heteroresistance of Omadacycline Against Clinical Staphylococcus aureus Isolates From China Reveal the Impact of Omadacycline Susceptibility by Branched-Chain Amino Acid Transport System II Carrier Protein, Na/Pi Cotransporter Family Protein, and Fibronectin-Binding Protein. Frontiers in microbiology, 10, 2546.

Band, V. I., Crispell, E. K., Napier, B. A., Herrera, C. M., Tharp, G. K., Vavikolanu, K., Pohl, J., Read, T. D., Bosinger, S. E., Trent, M. S., Burd, E. M., & Weiss, D. S. (2016). Antibiotic failure mediated by a resistant subpopulation in Enterobacter cloacae. Nature microbiology, 1(6), 16053.

Band, V. I., Hufnagel, D. A., Jaggavarapu, S., Sherman, E. X., Wozniak, J. E., Satola, S. W., Farley, M. M., Jacob, J. T., Burd, E. M., & Weiss, D. S. (2019). Antibiotic combinations that exploit heteroresistance to multiple drugs effectively control infection. Nature microbiology, 4(10), 1627–1635.

Band, V. I., Satola, S. W., Burd, E. M., Farley, M. M., Jacob, J. T., & Weiss, D. S. (2018). Carbapenem-Resistant Klebsiella pneumoniae Exhibiting Clinically Undetected Colistin Heteroresistance Leads to Treatment Failure in a Murine Model of Infection. mBio, 9(2), e02448-17.

Band, V. I., & Weiss, D. S. (2019). Heteroresistance: A cause of unexplained antibiotic treatment failure? PLoS pathogens, 15(6), e1007726.

Bari, M. S., Rahman, M. M., Persson, Y., Derks, M., Sayeed, M. A., Hossain, D., Singha, S., Hoque, M. A., Sivaraman, S., Fernando, P., Ahmad, I., Samad, A., & Koop,

G. (2022). Subclinical mastitis in dairy cows in south-Asian countries: a review of risk factors and etiology to prioritize control measures. Veterinary research communications, 46(3), 621–640.

Binh, C. T., Heuer, H., Kaupenjohann, M., & Smalla, K. (2008). Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS microbiology ecology, 66(1), 25–37.

Brauner, A., Fridman, O., Gefen, O., & Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nature reviews. Microbiology, 14(5), 320–330.

Brunn, A., Kadri-Alabi, Z., Moodley, A., Guardabassi, L., Taylor, P., Mateus, A. and Waage, J. (2022a). Characteristics and global occurrence of human pathogens harboring antimicrobial resistance in food crops: A scoping review. Frontiers in Sustainable Food Systems 6: 824714.

Brunn, A. A., Roustit, M., Kadri-Alabi, Z., Guardabassi, L., & Waage, J. (2022b). A Meta-Analysis to Estimate Prevalence of Resistance to Tetracyclines and Third Generation Cephalosporins in Enterobacteriaceae Isolated from Food Crops. Antibiotics (Basel, Switzerland), 11(10), 1424.

Bueno, I., Williams-Nguyen, J., Hwang, H., Sargeant, J. M., Nault, A. J., & Singer, R. S. (2018). Systematic Review: Impact of point sources on antibiotic-resistant bacteria in the natural environment. Zoonoses and public health, 65(1), e162–e184.

Call, D. R., Singer, R. S., Meng, D., Broschat, S. L., Orfe, L. H., Anderson, J. M., Herndon, D. R., Kappmeyer, L. S., Daniels, J. B., & Besser, T. E. (2010). blaCMY-2-positive IncA/C plasmids from Escherichia coli and Salmonella enterica are a distinct component of a larger lineage of plasmids. Antimicrobial agents and chemotherapy, 54(2), 590–596.

Callens, B., Cargnel, M., Sarrazin, S., Dewulf, J., Hoet, B., Vermeersch, K., Wattiau, P., & Welby, S. (2018). Associations between a decreased veterinary antimicrobial use and resistance in commensal Escherichia coli from Belgian livestock species (2011-2015). Preventive veterinary medicine, 157, 50–58.

Chantziaras, I., Boyen, F., Callens, B., & Dewulf, J. (2014). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. The Journal of antimicrobial chemotherapy, 69(3), 827–834.

Chen, Y., Hu, D., Zhang, Q., Liao, X. P., Liu, Y. H., & Sun, J. (2017). Efflux Pump Overexpression Contributes to Tigecycline Heteroresistance in Salmonella enterica serovar Typhimurium. Frontiers in cellular and infection microbiology, 7, 37.

Cheong, H. S., Kim, S. Y., Wi, Y. M., Peck, K. R., & Ko, K. S. (2019). Colistin Heteroresistance in Klebsiella Pneumoniae Isolates and Diverse Mutations of

PmrAB and PhoPQ in Resistant Subpopulations. Journal of clinical medicine, 8(9), 1444.

Collis, R. M., Burgess, S. A., Biggs, P. J., Midwinter, A. C., French, N. P., Toombs-Ruane, L., & Cookson, A. L. (2019). Extended-Spectrum Beta-Lactamase-Producing Enterobacteriaceae in Dairy Farm Environments: A New Zealand Perspective. Foodborne pathogens and disease, 16(1), 5–22.

Costa, M. C., Bessegatto, J. A., Alfieri, A. A., Weese, J. S., Filho, J. A., & Oba, A. (2017). Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. PloS one, 12(2), e0171642.

Cottell, J. L., Kanwar, N., Castillo-Courtade, L., Chalmers, G., Scott, H. M., Norby, B., Loneragan, G. H., & Boerlin, P. (2013). blaCTX-M-32 on an IncN plasmid in Escherichia coli from beef cattle in the United States. Antimicrobial agents and chemotherapy, 57(2), 1096–1097.

de Campos, J. L., Kates, A., Steinberger, A., Sethi, A., Suen, G., Shutske, J., Safdar, N., Goldberg, T., & Ruegg, P. L. (2021). Quantification of antimicrobial usage in adult cows and preweaned calves on 40 large Wisconsin dairy farms using dose-based and mass-based metrics. Journal of dairy science, 104(4), 4727–4745.

Dorado-García, A., Smid, J. H., van Pelt, W., Bonten, M. J. M., Fluit, A. C., van den Bunt, G., Wagenaar, J. A., Hordijk, J., Dierikx, C. M., Veldman, K. T., de Koeijer, A., Dohmen, W., Schmitt, H., Liakopoulos, A., Pacholewicz, E., Lam, T. J. G. M., Velthuis, A. G., Heuvelink, A., Gonggrijp, M. A., van Duijkeren, E., … Heederik, D. J. J. (2018). Molecular relatedness of ESBL/AmpC-producing Escherichia coli from humans, animals, food and the environment: a pooled analysis. The Journal of antimicrobial chemotherapy, 73(2), 339–347.

El-Halfawy, O. M., & Valvano, M. A. (2015). Antimicrobial heteroresistance: an emerging field in need of clarity. Clinical microbiology reviews, 28(1), 191–207.

Enne, V. I., Bennett, P. M., Livermore, D. M., & Hall, L. M. (2004). Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. The Journal of antimicrobial chemotherapy, 53(6), 958–963.

Estrada, A. L., Li, Y. Y., & Wang, A. (2012). Biodegradability enhancement of wastewater containing cefalexin by means of the electro-Fenton oxidation process. Journal of hazardous materials, 227-228, 41–48.

Falagas, M. E., Makris, G. C., Dimopoulos, G., & Matthaiou, D. K. (2008). Heteroresistance: a concern of increasing clinical significance? Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 14(2), 101–104.

Ganda, E. K., Bisinotto, R. S., Lima, S. F., Kronauer, K., Decter, D. H., Oikonomou, G., Schukken, Y. H., & Bicalho, R. C. (2016). Longitudinal metagenomic profiling of bovine milk to assess the impact of intramammary treatment using a third-generation cephalosporin. Scientific reports, 6, 37565.

Gelalcha, B. D., Ensermu, D. B., Agga, G. E., Vancuren, M., Gillespie, B. E., D'Souza, D. H., Okafor, C. C., & Kerro Dego, O. (2022). Prevalence of Antimicrobial

Resistant and Extended-Spectrum Beta-Lactamase-producing Escherichia coli in Dairy Cattle Farms in East Tennessee. Foodborne pathogens and disease, 19(6), 408–416.

Gibson, J. S., Morton, J. M., Cobbold, R. N., Filippich, L. J., & Trott, D. J. (2011). Risk factors for dogs becoming rectal carriers of multidrug-resistant Escherichia coli during hospitalization. Epidemiology and infection, 139(10), 1511–1521.

Goren, M. G., Carmeli, Y., Schwaber, M. J., Chmelnitsky, I., Schechner, V., & Navon-Venezia, S. (2010). Transfer of carbapenem-resistant plasmid from Klebsiella pneumoniae ST258 to Escherichia coli in patient. Emerging infectious diseases, 16(6), 1014–1017.

Grenda, T., Grenda, A., Domaradzki, P., Krawczyk, P., & Kwiatek, K. (2022). Probiotic Potential of Clostridium spp.-Advantages and Doubts. Current issues in molecular biology, 44(7), 3118–3130.

Harris P. N. (2015). Clinical management of infections caused by Enterobacteriaceae that express extended-spectrum β-lactamase and AmpC enzymes. Seminars in respiratory and critical care medicine, 36(1), 56–73. https://doi.org/10.1055/s-0034-1398387

Hasman, H., Mevius, D., Veldman, K., Olesen, I., & Aarestrup, F. M. (2005). beta-Lactamases among extended-spectrum beta-lactamase (ESBL)-resistant Salmonella from poultry, poultry products and human patients in The Netherlands. The Journal of antimicrobial chemotherapy, 56(1), 115–121.

Hayer, S. S., Casanova-Higes, A., Paladino, E., Elnekave, E., Nault, A., Johnson, T., Bender, J., Perez, A., & Alvarez, J. (2022). Global Distribution of Extended Spectrum Cephalosporin and Carbapenem Resistance and Associated Resistance Markers in Escherichia coli of Swine Origin - A Systematic Review and Meta-Analysis. Frontiers in microbiology, 13, 853810.

He, Y., Yuan, Q., Mathieu, J., Stadler, L., Senehi, N., Sun, R., Alvarez, P.J. (2020). Antibiotic resistance genes from livestock waste: Occurrence, dissemination, and treatment. NPJ Clean Water, 3, 4.

Hjort, K., Nicoloff, H., & Andersson, D. I. (2016). Unstable tandem gene amplification generates heteroresistance (variation in resistance within a population) to colistin in Salmonella enterica. Molecular microbiology, 102(2), 274–289.

Ikonomidis, A., Tsakris, A., Kantzanou, M., Spanakis, N., Maniatis, A. N., & Pournaras, S. (2008). Efflux system overexpression and decreased OprD contribute to the carbapenem heterogeneity in Pseudomonas aeruginosa. FEMS microbiology letters, 279(1), 36–39.

Jayol, A., Nordmann, P., Brink, A., & Poirel, L. (2015). Heteroresistance to colistin in Klebsiella pneumoniae associated with alterations in the PhoPQ regulatory system. Antimicrobial agents and chemotherapy, 59(5), 2780–2784.

Jo, J., & Ko, K. S. (2021). Tigecycline Heteroresistance and Resistance Mechanism in Clinical Isolates of Acinetobacter baumannii. Microbiology spectrum, 9(2), e0101021.

Koops, W.J.; Ekstrom, J.; Armstrong, D. (2018). 5.8 Report on Practical Strategies to Reduce Antimicrobial Use in Dairy Farming; EuroDairy.

Kouhsari, E., Sadeghifard, N., Khadiv, A., Sayadi, H., Amiriani, T., Ghafourian, S., Valadbeigi, H., & Krutova, M. (2022). Heteroresistance to clarithromycin and metronidazole in patients with a Helicobacter pylori infection: a systematic review and meta-analysis. Annals of clinical microbiology and antimicrobials, 21(1), 19.

Kuang, Q., He, D., Sun, H., Hu, H., Li, F., Li, W., Hu, G., Wu, H., & Yuan, L. (2020). R93P Substitution in the PmrB HAMP Domain Contributes to Colistin Heteroresistance in Escherichia coli Isolates from Swine. Antimicrobial agents and chemotherapy, 64(11), e01509-20.

Lee, J. Y., Choi, M. J., Choi, H. J., & Ko, K. S. (2015). Preservation of Acquired Colistin Resistance in Gram-Negative Bacteria. Antimicrobial agents and chemotherapy, 60(1), 609–612.

Levent, G., Schlochtermeier, A., Vinasco, J., Jennings, J., Richeson, J., Ives, S. E., Norman, K. N., Lawhon, S. D., Loneragan, G. H., & Scott, H. M. (2022). Long-Term Effects of Single-Dose Cephalosporin or Macrolide Use on the Prevalence of AmpC and Extended-Spectrum β-Lactamase Producing Escherichia coli in the Feces of Beef Cattle. Microorganisms, 10(10), 2071.

Li, W. R., Zhang, Z. Q., Liao, K., Wang, B. B., Liu, H. Z., Shi, Q. S., Huang, X. B., & Xie, X. B. (2022). Pseudomonas aeruginosa heteroresistance to levofloxacin caused by upregulated expression of essential genes for DNA replication and repair. Frontiers in microbiology, 13, 1105921.

Lima, S. F., Bicalho, M. L. S., & Bicalho, R. C. (2018). Evaluation of milk sample fractions for characterization of milk microbiota from healthy and clinical mastitis cows. PloS one, 13(3), e0193671.

Liu, H., Li, C., Liang, Z., Zhang, S., Yang, W., Ye, Y., Lin, Y., Chen, R., Zhou, H., Su, J. (2019). Moraxellaceae and Moraxella interact with the altered airway mycobiome in asthma. bioRxiv, 525113.

Lohsen, S., & Stephens, D. S. (2023). Inducible Mega-Mediated Macrolide Resistance Confers Heteroresistance in Streptococcus pneumoniae. Antimicrobial agents and chemotherapy, 67(3), e0131922.

Lowrance, T. C., Loneragan, G. H., Kunze, D. J., Platt, T. M., Ives, S. E., Scott, H. M., Norby, B., Echeverry, A., & Brashears, M. M. (2007). Changes in antimicrobial susceptibility in a population of Escherichia coli isolated from feedlot cattle administered ceftiofur crystalline-free acid. American journal of veterinary research, 68(5), 501–507.

Lu, Y., Liu, Y., Zhou, C., Liu, Y., Long, Y., Lin, D., Xiong, R., Xiao, Q., Huang, B., & Chen, C. (2022). Quorum sensing regulates heteroresistance in Pseudomonas aeruginosa. Frontiers in microbiology, 13, 1017707.

Ludden, C., Raven, K. E., Jamrozy, D., Gouliouris, T., Blane, B., Coll, F., de Goffau, M., Naydenova, P., Horner, C., Hernandez-Garcia, J., Wood, P., Hadjirin, N., Radakovic, M., Brown, N. M., Holmes, M., Parkhill, J., & Peacock, S. J. (2019). One Health Genomic Surveillance of Escherichia coli Demonstrates Distinct Lineages and Mobile Genetic Elements in Isolates from Humans versus Livestock. mBio, 10(1), e02693-18.

Ma, W., Sun, J., Yang, S., & Zhang, L. (2016). Epidemiological and clinical features for cefepime heteroresistant Escherichia coli infections in Southwest China. European journal of clinical microbiology & infectious diseases: official publication of the European Society of Clinical Microbiology, 35(4), 571–578.

Magdaleno, A., Saenz, M. E., Juárez, A. B., & Moretton, J. (2015). Effects of six antibiotics and their binary mixtures on growth of Pseudokirchneriella subcapitata. Ecotoxicology and environmental safety, 113, 72–78.

Moodley, A., & Guardabassi, L. (2009). Transmission of IncN plasmids carrying blaCTX-M-1 between commensal Escherichia coli in pigs and farm workers. Antimicrobial agents and chemotherapy, 53(4), 1709–1711.

Morand, B., & Mühlemann, K. (2007). Heteroresistance to penicillin in Streptococcus pneumoniae. Proceedings of the National Academy of Sciences of the United States of America, 104(35), 14098–14103.

Nicoloff, H., Hjort, K., Levin, B. R., & Andersson, D. I. (2019). The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. Nature microbiology, 4(3), 504–514.

Oliveira, L., Hulland, C., & Ruegg, P. L. (2013). Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. Journal of dairy science, 96(12), 7538–7549.

Penders, J., Stobberingh, E. E., Savelkoul, P. H., & Wolffs, P. F. (2013). The human microbiome as a reservoir of antimicrobial resistance. Frontiers in microbiology, 4, 87.

Pinzón-Sánchez, C., Cabrera, V. E., & Ruegg, P. L. (2011). Decision tree analysis of treatment strategies for mild and moderate cases of clinical mastitis occurring in early lactation. Journal of dairy science, 94(4), 1873–1892.

Rawat, D., & Nair, D. (2010). Extended-spectrum β-lactamases in Gram Negative Bacteria. Journal of global infectious diseases, 2(3), 263–274.

Rahman, M., Alam, M. U., Luies, S. K., Kamal, A., Ferdous, S., Lin, A., Sharior, F., Khan, R., Rahman, Z., Parvez, S. M., Amin, N., Hasan, R., Tadesse, B. T., Taneja, N., Islam, M. A., & Ercumen, A. (2021). Contamination of Fresh Produce with Antibiotic-Resistant Bacteria and Associated Risks to Human Health: A Scoping

Review. International journal of environmental research and public health, 19(1), 360.

Ram, S., & Goulian, M. (2013). The architecture of a prototypical bacterial signaling circuit enables a single point mutation to confer novel network properties. PLoS genetics, 9(8), e1003706.

Rodríguez, I., Barownick, W., Helmuth, R., Mendoza, M. C., Rodicio, M. R., Schroeter, A., & Guerra, B. (2009). Extended-spectrum {beta}-lactamases and AmpC {beta}-lactamases in ceftiofur-resistant Salmonella enterica isolates from food and livestock obtained in Germany during 2003-07. The Journal of antimicrobial chemotherapy, 64(2), 301–309.

Ruegg, P.L., Erskine, R.D. (2020). Mammary Gland Health and Disorders. In Large Animal Veterinary Internal Medicine, 6th ed.; Smith, B.P.,Ed.; Elsevier: Amsterdam, The Netherlands, pp. 1018–1150.

Shintani, M., Sanchez, Z. K., & Kimbara, K. (2015). Genomics of microbial plasmids: classification and identification based on replication and transfer systems and host taxonomy. Frontiers in microbiology, 6, 242.

Singh, N. S., Singhal, N., & Virdi, J. S. (2018). Genetic Environment of blaTEM-1, blaCTX-M-15, blaCMY-42 and Characterization of Integrons of Escherichia coli Isolated From an Indian Urban Aquatic Environment. Frontiers in microbiology, 9, 382.

Sivapalasingam, S., Friedman, C. R., Cohen, L., & Tauxe, R. V. (2004). Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. Journal of food protection, 67(10), 2342–2353.

Subbiah, M., Top, E. M., Shah, D. H., & Call, D. R. (2011). Selection pressure required for long-term persistence of blaCMY-2-positive IncA/C plasmids. Applied and environmental microbiology, 77(13), 4486–4493.

Sorg, R. A., & Veening, J. W. (2015). Microscale insights into pneumococcal antibiotic mutant selection windows. Nature communications, 6, 8773.

Salgado-Caxito, M., Moreno-Switt, A. I., Paes, A. C., Shiva, C., Munita, J. M., Rivas, L., & Benavides, J. A. (2021). Higher Prevalence of Extended-Spectrum Cephalosporin-Resistant Enterobacterales in Dogs Attended for Enteric Viruses in Brazil Before and After Treatment with Cephalosporins. Antibiotics (Basel, Switzerland), 10(2), 122.

Schukken, Y. H., Bennett, G. J., Zurakowski, M. J., Sharkey, H. L., Rauch, B. J., Thomas, M. J., Ceglowski, B., Saltman, R. L., Belomestnykh, N., & Zadoks, R. N. (2011). Randomized clinical trial to evaluate the efficacy of a 5-day ceftiofur hydrochloride intramammary treatment on nonsevere gram-negative clinical mastitis. Journal of dairy science, 94(12), 6203–6215.

Sheedy, D. B., Okello, E., Williams, D. R., Precht, K., Cella, E., Lehenbauer, T. W., & Aly, S. S. (2021). Effect of Antimicrobial Treatment on the Dynamics of Ceftiofur Resistance in Enterobacteriaceae from Adult California Dairy Cows. Microorganisms, 9(4), 828.

Van Boeckel, T. P., Pires, J., Silvester, R., Zhao, C., Song, J., Criscuolo, N. G., Gilbert, M., Bonhoeffer, S., & Laxminarayan, R. (2019). Global trends in antimicrobial resistance in animals in low- and middle-income countries. Science (New York, N.Y.), 365(6459), eaaw1944.

Wang, Z., Chen, Q., Zhang, J., Yan, H., Chen, Y., Chen, C., & Chen, X. (2021). High prevalence of unstable antibiotic heteroresistance in cyanobacteria causes resistance underestimation. Water research, 202, 117430.

Warnes, S. L., Highmore, C. J., & Keevil, C. W. (2012). Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: implications for public health. mBio, 3(6), e00489-12.

Weinroth, M. D., Scott, H. M., Norby, B., Loneragan, G. H., Noyes, N. R., Rovira, P., Doster, E., Yang, X., Woerner, D. R., Morley, P. S., & Belk, K. E. (2018). Effects of Ceftiofur and Chlortetracycline on the Resistomes of Feedlot Cattle. Applied and environmental microbiology, 84(13), e00610-18.

Werngren, J., Mansjö, M., Glader, M., Hoffner, S., & Davies Forsman, L. (2021). Detection of Pyrazinamide Heteroresistance in Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy, 65(9), e0072021.

WHO Regional Office for Europe and European Centre for Disease Prevention and Control. Surveillance of antimicrobial resistance in Europe, 2020 data. In Executive Summary; WHO Regional Office for Europe: Copenhagen, Denmark, 2021.

Wu, H., Nguyen, Q. D., Tran, T. T. M., Tang, M. T., Tsuruta, T., & Nishino, N. (2019). Rumen fluid, feces, milk, water, feed, airborne dust, and bedding microbiota in dairy farms managed by automatic milking systems. Animal science journal = Nihon chikusan Gakkaiho, 90(3), 445–452.

Wu, H., Wang, Y., Dong, L., Hu, H., Meng, L., Liu, H., Zheng, N., & Wang, J. (2020). Microbial Characteristics and Safety of Dairy Manure ComPosting for Reuse as Dairy Bedding. Biology, 10(1), 13.

Xiong, W., Wang, Y., Sun, Y., Ma, L., Zeng, Q., Jiang, X., Li, A., Zeng, Z., & Zhang, T. (2018). Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. Microbiome, 6(1), 34.

Castanheira, M., Simner, P. J., & Bradford, P. A. (2021). Extended-spectrum β-lactamases: an update on their characteristics, epidemiology and detection. JAC-antimicrobial resistance, 3(3), dlab092.

Xu, Y., Zheng, X., Zeng, W., Chen, T., Liao, W., Qian, J., Lin, J., Zhou, C., Tian, X., Cao, J., & Zhou, T. (2020). Mechanisms of Heteroresistance and Resistance to Imipenem in Pseudomonas aeruginosa. Infection and drug resistance, 13, 1419–1428.

Yu, M., Mu, C., Zhang, C., Yang, Y., Su, Y., & Zhu, W. (2018). Marked Response in Microbial Community and Metabolism in the Ileum and Cecum of Suckling Piglets After Early Antibiotics Exposure. Frontiers in microbiology, 9, 1166.

Zeineldin, M., Megahed, A., Burton, B., Blair, B., Aldridge, B., & Lowe, J. F. (2019). Effect of Single Dose of Antimicrobial Administration at Birth on Fecal Microbiota Development and Prevalence of Antimicrobial Resistance Genes in Piglets. Frontiers in microbiology, 10, 1414.

Zheng, J. X., Lin, Z. W., Sun, X., Lin, W. H., Chen, Z., Wu, Y., Qi, G. B., Deng, Q. W., Qu, D., & Yu, Z. J. (2018). Overexpression of OqxAB and MacAB efflux pumps contributes to eravacycline resistance and heteroresistance in clinical isolates of Klebsiella pneumoniae. Emerging microbes & infections, 7(1), 139.

Appendix

Scientific publications

Scientific publications

FIRST AUTHOR

Dong L, Meng L, Liu H, Wu H, Hu H, Zheng N, Wang J, Schroyen M. Effect of therapeutic administration of β-lactam antibiotics on the bacterial community and antibiotic resistance patterns in milk. J Dairy Sci. 2021 Jun;104(6):7018-7025.

Dong L, Meng L, Liu H, Wu H, Schroyen M, Zheng N, Wang J. Effect of Cephalosporin Treatment on the Microbiota and Antibiotic Resistance Genes in Feces of Dairy Cows with Clinical Mastitis. Antibiotics (Basel). 2022 Jan 17;11(1):117.

Dong L, Meng L, Liu H, Wu H, Schroyen M, Zheng N, Wang J. The study on the heteroresistance to cephalosporins of Staphylococcus in milk from mastitis suffering cows. (To be submitted).

Liu H, **Dong L**, Zhao Y, Meng L, Wang J, Wang C, Zheng N. Antimicrobial Susceptibility, and Molecular Characterization of Staphylococcus aureus Isolated from Different Raw Milk Samples in China. Front Microbiol. 2022 May 13;13: 840670. (co-first authors).

CO-AUTHOR

Wu, H.; Wang, Y.; **Dong, L**.; Hu, H.; Meng, L.; Liu, H.; Zheng, N.; Wang, J. Microbial Characteristics and Safety of Dairy Manure ComPosting for Reuse as Dairy Bedding. Biology 2021, 10, 13.

Meng L, Zhang R, **Dong L**, Hu H, Liu H, Zheng N, Wang J, Cheng J. Characterization and spoilage potential of Bacillus cereus isolated from farm environment and raw milk. Front Microbiol. 2022 Sep 14;13:940611.