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# Genetic diversity and molecular epidemiology of *Streptococcus uberis* in high-prevalence mastitis herds

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## ABSTRACT

*Streptococcus uberis* is one of the most frequently isolated bacterial species from mastitis cows and presents significant control challenges due to its high genetic diversity. This study aimed to determine genetic variations of *Strep. uberis* causing mastitis across 3 high-prevalence farms (farm A, B, and C). Isolates were obtained from different longitudinal studies and were used to study relationships between virulence factors (VF), antimicrobial resistance (AMR), and strain diversity. Data on farm management and the mastitis control policy were collected during longitudinal collection of milk samples and revealed different policies among the 3 farms. Among all available *Strep. uberis* isolates, strains were selected based on the characteristics of infection episodes, choosing a maximum of 2 isolates per episode. Selected isolates were confirmed using MALDI-TOF and subsequently used for whole-genome sequencing (WGS). All VF and AMR genes were categorized into the core genes, as present in all isolates; the accessory gene, as present in more than 50% of all isolates; and the unique genes for the genes that were present in less than 50% of all isolates. Accessory genes and unique genes were used to determine the relationships between VF and AMR using the Fisher exact chi-squared tests. The WGS results from a total of 138 *Strep. uberis* isolates, obtained from 92 episodes, revealed 7 distinct phylogroups (I to VII) and 32 gene patterns. Farm B, the only farm with long-time dairy experience and managed solely by experienced dairy farmers, exhibited the highest genetic diversity. In contrast, the other farms, dominated by persistent *Strep. uberis* IMI, showed fewer dominant patterns and lower diversity. A lower AMR prevalence in farm C (10.7%, 6/56) was associated with lower antibiotic use, as antimicrobial usage must be authorized by a local veterinarian, but the farmers of farms A and B designed their own use. In addition, both phylogenetic and farm factors revealed a significant association between virulence and AMR . From all identified 35 VF genes and 16 AMR genes, the core pattern included 21 core genes and 8 accessory genes from the VF genes, whereas the unique genes contained 6 VF and all 16 AMR genes. The results revealed that an increase in the number of AMR genes was associated with greater virulence diversity. Significant correlations were observed with overall VF gene presence or absence (r = 0.437), as well as with the absence of VF genes from the core pattern (R = 0.523). No AMR genes were detected in patterns without VF variations from the core pattern. Furthermore, a significant correlation was demonstrated between AMR genes and specific VF genes, notably with the presence of *cfu* and the absence of *hasA*. Thus, these findings highlight the complex relationship between virulence factors and resistance genes, which are potentially influenced by factors such as farm management practices and bacterial traits. This underscores the need for further studies that should be specific to each mastitis scenario, enabling the development of effective control strategies to reduce the impact of *Strep. uberis* mastitis.

**Key words:** mastitis, *Streptococcus uberis*, virulence factor, antimicrobial resistance

## INTRODUCTION

Mastitis is a prevalent and costly disease in dairy cows, leading to reduced animal health, reduction in welfare of affected animals and increased antibiotic use (Gussmann et al., 2019; Gonçalves et al., 2022). *Streptococcus uberis* is one of the major pathogens, responsible for both sub-

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

clinical and clinical mastitis in cattle worldwide (Tomazi et al., 2019). This bacterium is primarily transmitted via the environment, frequently isolated from various environmental sources in dairy farms, whereas cow-to-cow transmission may occur in herds with inadequate prevention (Rato et al., 2013). Several studies have demonstrated a high diversity of *Strep. uberis* IMI which, regardless of their transmission characteristics, can be both transient and persistent, with infection durations ranging from a few days to several months (Pullinger et al., 2007; Leelahapongsathon et al., 2016). The genetic diversity of mastitis pathogens has been linked to variations in management practices, including treatment protocols, farm policies, and sources of transmission. Such associations have been demonstrated in other pathogens, including *Staphylococcus aureus* (Middleton, 2013), *Klebsiella pneumoniae* (Cheng et al., 2021), and *Listeria monocytogenes* (Terentjeva et al., 2021), emphasizing the critical role of farm-specific management strategies in shaping pathogen diversity.

*Streptococcus uberis* has a varied cure rate from high to low cure rates for environmental to contagious *Strep. uberis*, respectively (Leelahapongsathon et al., 2020), compared with other mastitis pathogens because of its capability to survive intracellularly in the udder due to acquiring a wide array of virulence factors (**VF**; Tomazi et al., 2019; Fessia and Odierno, 2022) and the presence of antimicrobial resistance (**AMR**). This bacterium is a genetically highly variable with different levels of virulence. Despite its genetic diversity, most *Strep. uberis* strains share a core set of highly conserved virulence genes such as *oppF*, *pauA*, *sua*, *hasC* that are strongly associated with IMI pathogenesis (Perrig et al., 2015; Calonzi et al., 2020; Zouharova et al., 2022b). However, different *Strep. uberis* strains also differ in possession of the virulence-associated genes such as *cfu*, *hasA*, *hasB*, and *hylB* (which are frequently detected in *Strep. uberis* isolates; Shabayek and Spellerberg, 2018; Zhang et al., 2020; Zouharova et al., 2022b) and strain-specific VF genes, such as the *cps* gene cluster (Su et al., 2021). For example, prevalence of *cfu* gene ranged from 19% in Poland to 35% in Thailand and 77% in Argentina (Reinoso et al., 2011; Kaczorek et al., 2017; Boonyayatra et al., 2018).

During IMI, bacteria have modified their virulence to adapt to the host defense systems (Mayer et al., 2021). Virulence mechanisms are necessary for pathogenesis, and the development of AMR increasingly enables pathogenic bacteria to withstand antimicrobial treatment. Consequently, both factors can contribute to bacterial adaptation and survival in the udder micro-environment (Beceiro et al., 2013). In many countries, the preventive use of antibiotics has been limited; however, antibiotics are still used to prevent and treat *Strep. uberis* mastitis in dairy cows (El-Sayed and Kamel, 2021; Zamojska et al., 2021). However, AMR in livestock has become a serious global issue, with Thailand ranking third worldwide in AMR prevalence (Mulchandani et al., 2023). The evolution of bacteria resistant to multiple antibiotics arises from genetic variations that enable rapid adaptation to changing environmental conditions (Guo et al., 2021). Over time, *Strep. uberis* has exhibited increasing resistance to antimicrobials. Its resistance to macrolides, lincosamides, and tetracyclines has been documented (de Jong et al., 2018; Käppeli et al., 2019; Reyes et al., 2019). Significant associations between AMR and VF have also been reported in various bacterial species (Cepas and Soto, 2020), such as *Escherichia coli* (Do et al., 2022) and *Klebsiella pneumoniae* (Kawser and Shamsuzzaman, 2022; Kumaran et al., 2022).

Despite several investigations, there remains no conclusive evidence to determine the association between AMR and VF, as well as the factors driving genetic variation in *Strep. uberis* under different environmental conditions and phylogenetic lineages. This research focused therefore on the genetic variation of *Strep. uberis* in farms with high mastitis prevalence, particularly in relation to poor hygiene and inadequate mastitis control programs with relatively frequent use of antimicrobials. The advance of knowledge about the genetic variation associated with *Strep. uberis* causing mastitis associated with farm management practices can contribute to the developing of efficient strategies for prevention and control of this pathogen in dairy herds. Therefore, the objectives of this study were to (1) examine the prevalence and genetic variability of VF and AMR genes, (2) identify the factors driving these variations and the presence of genetic correlations, and (3) explore the relationship between VF and AMR genes. This study aims to compare the gene variations in virulence and AMR of *Strep. uberis* in farms with high mastitis prevalence and different management practices.

## MATERIALS AND METHODS

*Streptococcus uberis* isolates were selected from the *Strep. uberis* stock collected from 2 previous mastitis longitudinal studies (Leelahapongsathon et al., 2020; Srithanasuwan et al., 2022), stored at Chiang Mai University in Thailand. Briefly, these longitudinal studies reported on aseptically collected milk samples from all quarters with a specified frequency and duration (weekly, monthly) in 3 smallholder dairy farms with a high prevalence of mastitis (Table 1). The collected samples were used for bacterial identification by MALDI-TOF and stored at −80°C until selection. Intramammary infection was defined based on the criteria established by Leelahapongsathon et al. (2020) and Zadoks et al. (2003). Briefly, a quarter

**Table 1.** Farm characteristics and management

| Attribute | Farm A | Farm B | Farm C |
|---|---|---|---|
| Farm characteristic | | | |
| Farm experience (>5 yr) | No | Yes | No |
| Owner-managed | No | Yes | Yes |
| Labor force (2 laborers) | Yes | Yes | Yes |
| Number of lactating cows | 27 | 31 | 30 |
| Barn type[1] | Tiestall | Tiestall | Freestall |
| Good ventilation housing | No | No | Yes |
| Milking machines[2] (n) | 2 | 4 | 2 |
| Mastitis control policy | | | |
| Use of individual paper towels | No | Yes | No |
| Immediate postmilking teat disinfection | No | No | Yes |
| Segregating infected cows | No | No | No |
| Milking known infected cows last | No | Yes | Yes |
| Routine culling persistent IMI or recurrent clinical mastitis | No | Sometimes | Sometimes |
| Decision criteria for antibiotic treatment of clinical mastitis | Based on severity and number of mastitis quarters | Based on severity and days in milk | Based on severity |
| Authorization of antibiotic use | Workers | Owner | Local Veterinarians |
| Major antibiotics used on the farm | Oxytetracycline, gentamicin, penicillin, streptomycin | Oxytetracycline, penicillin, streptomycin, cloxacillin, gentamicin | Oxytetracycline, gentamicin, cloxacillin, enrofloxacin |
| Mastitis status at the start of the study and sample collection | | | |
| BTSCC[3] (cells/mL) | >1,000,000 | >1,000,000 | >800,000 |
| Prevalence of *Streptococcus uberis* IMI at the start of the study (% at cow level) | 33.3 | 67.7 | 56.7 |
| Sample collection interval | Monthly | Weekly | Monthly |
| Sampling duration | 10 mo | 10 wk | 4 mo |

[1]All barns had concrete floor.

[2]All farms milked twice daily with the bucket-type milking machine.

[3]Bulk tank somatic cell counts.

was considered infected with *Strep. uberis* if ≥1,000 cfu/mL of the pathogen was cultured from a single sample, ≥500 cfu/mL was cultured from 2 of 3 consecutive milk samples, ≥100 cfu/mL was cultured from 3 consecutive milk samples, or ≥100 cfu/mL was cultured from a clinical case sample. Data on the study design, farms, and mastitis management during the longitudinal studies are presented in Table 1. The 3 herds were housed in tiestall facilities with a concrete floor and consisted of ~27 to 31 crossbred Holstein Friesian lactating cows. Cows were milked twice daily. Despite varying farm management practices and antimicrobial regulations, all farms demonstrated relatively poor husbandry and milking practices without regular maintenance of the milking machines. In general, antibiotics were used by local veterinarians for the treatment of severely sick cows, except in cases of mastitis and lameness, where antibiotics could be administered by workers (farm A), owner (farm B), or veterinarians (farm C). This resulted in differences in the average frequency of antibiotic usage, reported as >4 times per lactation, 2 to 4 times per lactation, and <2 times per lactation, respectively. In addition, it was noted that suboptimal doses of antibiotics are often used in these treatments (farms A and B).

To capture the genetic diversity of bacterial isolates, representatives of all IMI episodes were selected. Bacterial selection criteria were based on the observed infection episode. The duration of an episode of *Strep. uberis* was determined when the *Strep. uberis* strain continued to be identified with the same pulsed-field gel electrophoresis type (farm A; Leelahapongsathon et al., 2020) or the same species (Farms B and C). The defined bacterial episode ended when a change to other strains, other bacteria, or a negative result for at least 2 specified periods was observed. The episodes were determined as persistent episodes when the infection duration was longer than 1 mo, whereas a shorter infection duration was defined as transient. Regardless of infection duration, transient episodes with only a 1-time positive sample were also selected. In cases of persistent *Strep. uberis* infections, 2 bacterial isolates from both the beginning and the end of the infection episode were used.

### Bacterial Preparation and DNA Extraction

All selected frozen *Strep. uberis* isolates were regrown on blood agar (Himedia, Mumbai, India) with 5% bovine blood and incubated at 37°C for 24 h. Genomic DNA

from each strain was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) on the QIAcube Connect, according to the manufacturer's instructions. Quantification was done by measuring the samples with the NanoDrop Microvolume Spectrophotometers (ThermoFisher Scientific, Waltham, MA).

### Genome Sequencing, De Novo Assembly, and Annotation

The DNA samples were sequenced using the Illumina NovaSeq X Plus platform (Illumina, San Diego, CA) with a 2 × 150 bp paired-end configuration. The adapter sequences were trimmed using the Trimmomatic tool (version 0.39; Bolger et al., 2014). For the trimmed reads, the online program FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to plot quality score (Phred quality score ≥20) and sequence length distribution and sequences were assembled using SPAdes (http://bioinf.spbau.ru/spades). Genome assemblies were annotated using the Prokka pipeline (version 1.13; Seemann, 2014). The whole-genome sequencing results of all *Strep. uberis* isolates are publicly available in the supplemental materials (Supplemental Table S1, see Notes), the Sequence Read Archive database (https://www.ncbi.nlm.nih.gov/sra/docs/), and the National Center for Biotechnology Information GenBank (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=1349) under BioProject accession number PRJNA883037.

### Genome Analysis

ABRicate version 1.0.1 (https://github.com/tseemann/abricate) was used with the VF database Chen et al., 2016) and a custom gene list, the SuPVDB (Vezina et al., 2021). All isolate genome assemblies were screened for AMR genes using ResFinder (Zankari et al., 2012) and the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013) (accessed 4/5/2024). ABRicate used a default threshold of 80% identity and 80% coverage for both virulence and resistance genes. Multidrug resistance was defined as *Strep. uberis* isolates that harbored resistance genes for 3 or more different antimicrobials (Magiorakos et al., 2012). Additionally, the mobile genetic elements were identified using the Mobile Element Finder tool v1.0.3 (https://cge.food.dtu.dk/services/MobileElementFinder/, accessed on 13/3/2025).

### SNP Phylogeny of Whole-Genome Sequences

The core SNP tree was constructed using PanTools v3. To identify SNPs, all the input sequences were mapped to the *Strep. uberis* 0140J genome as reference (GCF_000009545.1). First, single copy groups were aligned in 2 consecutive rounds by multiple alignment using fast Fourier transform. Parsimony informative positions from the nucleotide alignments were concatenated into a single contiguous sequence per genome from which IQ-tree generated a maximum likelihood tree with default parameters. Finally, the Newick-formatted tree files then were visualized with Interactive Tree Of Life (Letunic and Bork, 2019). Clade-clustering based on pairwise distances was used to categorize isolates into several phylogroups (Pardi and Gascuel, 2012).

### Statistical Analysis

Data were analyzed using SAS University Edition (SAS Institute Inc., Cary, NC). The phylogroups were identified based on genetic relationships and their VF-AMR patterns were defined by the identical patterns of VF and AMR genes in each phylogroup on each farm. The percentage of the specified genes on all isolates was used to determine the gene types, where 100% presence was considered a core gene, >50% presence was considered an accessory gene, and <50% presence was considered a unique gene, and the core pattern included the core genes and accessory genes. Gene variations were defined as any deviation from the core pattern, including virulence gene presence and absence. The association between phylogroups, farms, and genetic variation in individual genes (only those with presence or absence of a gene in more than 10 isolates) was evaluated using the Fisher's exact chi-squared test. The Spearman rank correlation was used to analyze the correlation between VF and AMR variations. Additionally, the association of individual VF and AMR genes was analyzed using the Fisher's exact test. The false discovery rate (**FDR**) procedure of Benjamini and Hochberg (1995) was used to correct for multiple hypothesis testing (FDR = 0.05; Richards et al., 2011). For all analyses, statistical significance was set at $P < 0.05$.

## RESULTS

### General Genome Features and MGE

The genome lengths ranged from 1,891,574 to 2,156,106 bp, with guanine-cytosine contents of 35.9% to 36.5%. Mobile genetic elements (**MGE**) analysis revealed that 114 isolates from farm A (n = 28), B (n = 42), and C (n = 44) carried plasmids. Most *Strep. uberis* isolates (n = 112) harbored plasmid repUS55, whereas one isolate from farm B carried repUS50 and repUS47, and 2 isolates from farm C carried Col440I, typically found in *Enterobacteriaceae*. A total of 46 MGE were detected in 44 isolates, distributed across farm A (n = 21), B (n = 17), and C (n = 6). Thirty-six MGE were linked to 1 to 2

AMR genes, including ant(6)-Ia (n = 32), tet(M) (n = 3), and tet(S) (n = 3).

### Phylogenetic and Distribution of Virulence and AMR Genes

A total of 138 *Strep. uberis* isolates were collected from 3 high-prevalence farms: farm A (n = 29), farm B (n = 53), and farm C (n = 56). These isolations originated from 45 cows (10, 21, and 14 cows, respectively), 91 quarters (19, 40, and 32 quarters, respectively), and 92 episodes of IMI (20, 40, and 32 episodes, respectively). Based on genetic relationship, the genome-wide SNP-based phylogenetic tree clustered the 138 isolates into 7 major phylogroups (I to VII), as shown in Figure 1. The distribution of isolates among the phylogroups was as follows: phylogroup I (1 isolate), II (50 isolates), III (27 isolates), IV (28 isolates), V (4 isolates), VI (23 isolates), and VII (5 isolates). Phylogroups I and II each showed only one VF-AMR gene pattern. Phylogroup IV had the highest number of VF-AMR patterns compared with other groups, which had 1 to 2 patterns each. Eleven distinct VF-AMR patterns were identified. Some isolates with patterns 13, 17, 19, 20, and 21 (n = 6, 2, 4, 3, and 7 isolates, respectively) shared the same VF-AMR gene pattern, and 6 isolates exhibited unique VF-AMR gene profiles.

As shown in Figure 1, 21 of the 38 VF genes were identified as core genes, including biofilm putative glycosyltransferase, *hasC*, *lmb*, *sclB*, *sua*, *fbpS*, *srtA*, *fbp54*, surface-anchored protein, *mtsB*, *scaR*, *mtuA*, *oppF*, *pauA*, *mga*, *scpA*, *lbp*, *cyG*, *cylA*, *cps4A*, and *emm*. Accessory VF genes, present in more than 57% of isolates, included *cpsB*, *cpsC*, *cps4D*, *cps4E*, *cpsJ*, *hasB*, *hylB*, and *cfu*. Unique VF genes, found in 7% to 40% of isolates, included *cps4H*, *cps4K/cps4L*, *cpsM*, *hasA*, and *bca*. All AMR genes were classified as unique genes. The proportion of isolates harboring at least one AMR gene was 79.3% (23/29) in farm A, 98.1% (52/53) in farm B, and 10.7% (6/56) in farm C. Multidrug resistance, resistant to ≥3 classes of antimicrobials, was observed in 29 isolates (~21%). In addition, none of the isolates from phylogroups I and II contained any AMR genes, whereas all isolates from phylogroups IV, V, VI, and VII contained at least one AMR gene.

### VF-AMR Patterns and Their Association with Phylogroups and Farms

The distribution of VF-AMR genes across isolates and patterns among phylogroups and farms is illustrated in Figure 2. The numbers of identical isolates and patterns based on gene variation within each phylogroup and farm were as follows: phylogroup I (1 pattern, 1 isolate), II (1 pattern, 50 isolates), III (8 patterns, 6, 1, 13, 1, 1, 3, 1, 1 isolates per pattern), IV (11 patterns, 1, 1, 6, 1, 1, 1, 2, 1, 4, 3, 7 isolates per pattern), V (4 patterns, 1 isolate per pattern), VI (4 patterns, 20, 1, 1, 1 isolates per pattern), and VII (4 patterns, 1, 1, 1, 2 isolates per pattern). Only phylogroup I exhibited the core gene pattern. Phylogroups III and IV showed substantial genetic diversity. Additionally, 4 distinct patterns were identified in phylogroups V to VII, each characterized by unique VF and AMR gene profiles.

To present the observed genetic diversity, accessory and unique genes were analyzed in relation to specific phylogroups (I to VII) and farms (A, B, and C), based on the percentage of patterns (Table 2) and isolates showing gene variations. Overall, most variations in VF and AMR genes and their respective groups or classes were significantly associated with specific phylogroups at pattern level ($P < 0.05$). However, some individual virulence genes such as *cps4K/cps4L* and AMR genes such as *aad(6)*, *APH(3')-IIIa*, *erm(B)*, *Isa(E)*, and *lnu(B)* did not show significant associations with phylogroups at the pattern level. No significant associations with farms were observed at the pattern level for either virulence or resistance genes. Furthermore, analysis of the relationship between phylogenetic groups and farms revealed a significant relationship (n = 32, $P = 0.02$).

### Relationship Between Virulence and AMR

The number of patterns harboring AMR genes and related to the presence or absence of virulence genes, excluding the core genes, is depicted in Figure 3. In Figure 3A, patterns with fewer virulence genes or absence of accessory genes from the core pattern exhibited higher AMR prevalence. A negative correlation was identified between the number of accessory VF genes and patterns harboring AMR, particularly for tetracycline ($P < 0.06$), lincosamides ($P < 0.05$), and macrolide resistance. The highest AMR gene presence was observed when accessory VF genes lost ≥2 genes. For unique VF genes, a similar negative relationship was found, where patterns with more unique VF genes tended to harbor fewer AMR genes, except for tetracycline ($P = 0.03$). Patterns without unique genes showed only a 7.41% prevalence of aminoglycoside resistance.

Figure 3B illustrated the Spearman rank correlation between virulence gene diversity compared with the core pattern and the presence or absence of AMR genes. Significant correlations were observed with overall VF gene deviations (R = 0.437, $P = 0.009$) and the absence of VF genes from the core pattern (R = 0.523, $P = 0.001$). Additionally, a significant correlation was found between total VF gene deviations and AMR ($P = 0.005$, Figure 3B). No AMR genes were detected in patterns without VF devia-

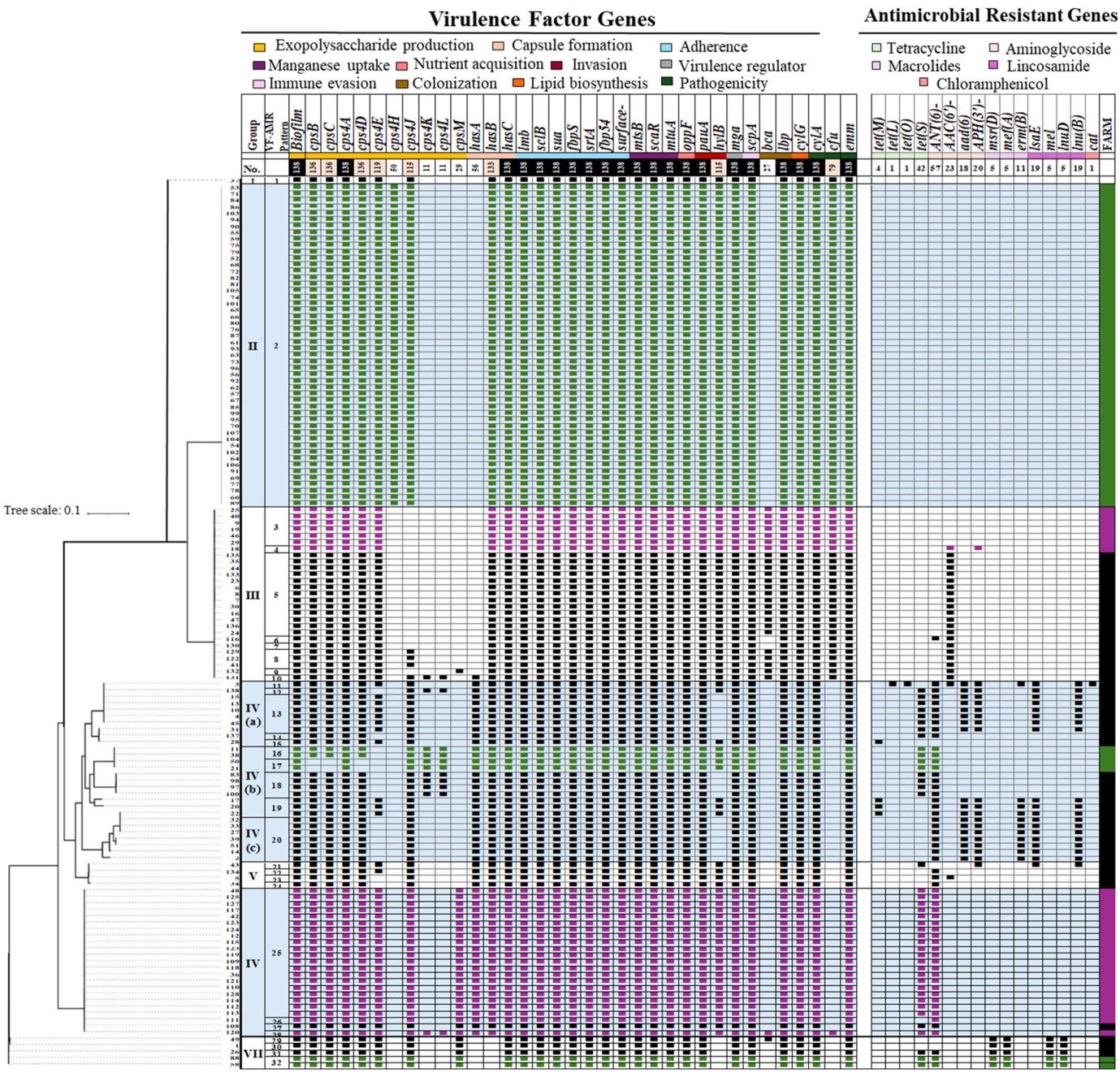



**Figure 1.** Phylogenetic analysis and distribution of the presence of virulence factor genes (VF) and antimicrobial resistance genes (AMR). A core SNP tree showing the phylogenetic structure of 138 *Streptococcus uberis* isolates from mastitis cases. The SNP tree was constructed based on the whole-genome alignment using Pantools V3. The scale bar represents the number of nucleotide substitutions per site. In the heatmap, column 1 displays the phylogenetic groups (I–VII), and column 2 lists the isolate ID. The right matrix panel shows the presence or absence of VF and AMR genes, with colored boxes indicating gene presence, varied by farm, and blank boxes representing gene absence. The number of isolates carrying each gene is shown at the top of the table. Black boxes denote core genes present in all isolates. The last column represents the farms: farm A (purple), farm B (black), and farm C (green).

tions from the core pattern. Patterns with at least 2 AMR genes were most commonly associated with the loss of 3 to 4 or ≥5 VF genes, highlighting the link between high virulence variation and increased AMR acquisition.

Meanwhile, individual AMR genes also had a strong association with some VF genes (Figure 3C). The gene *cfu* exhibited a significant association with multiple VF and AMR genes in the current study ($P < 0.05$),

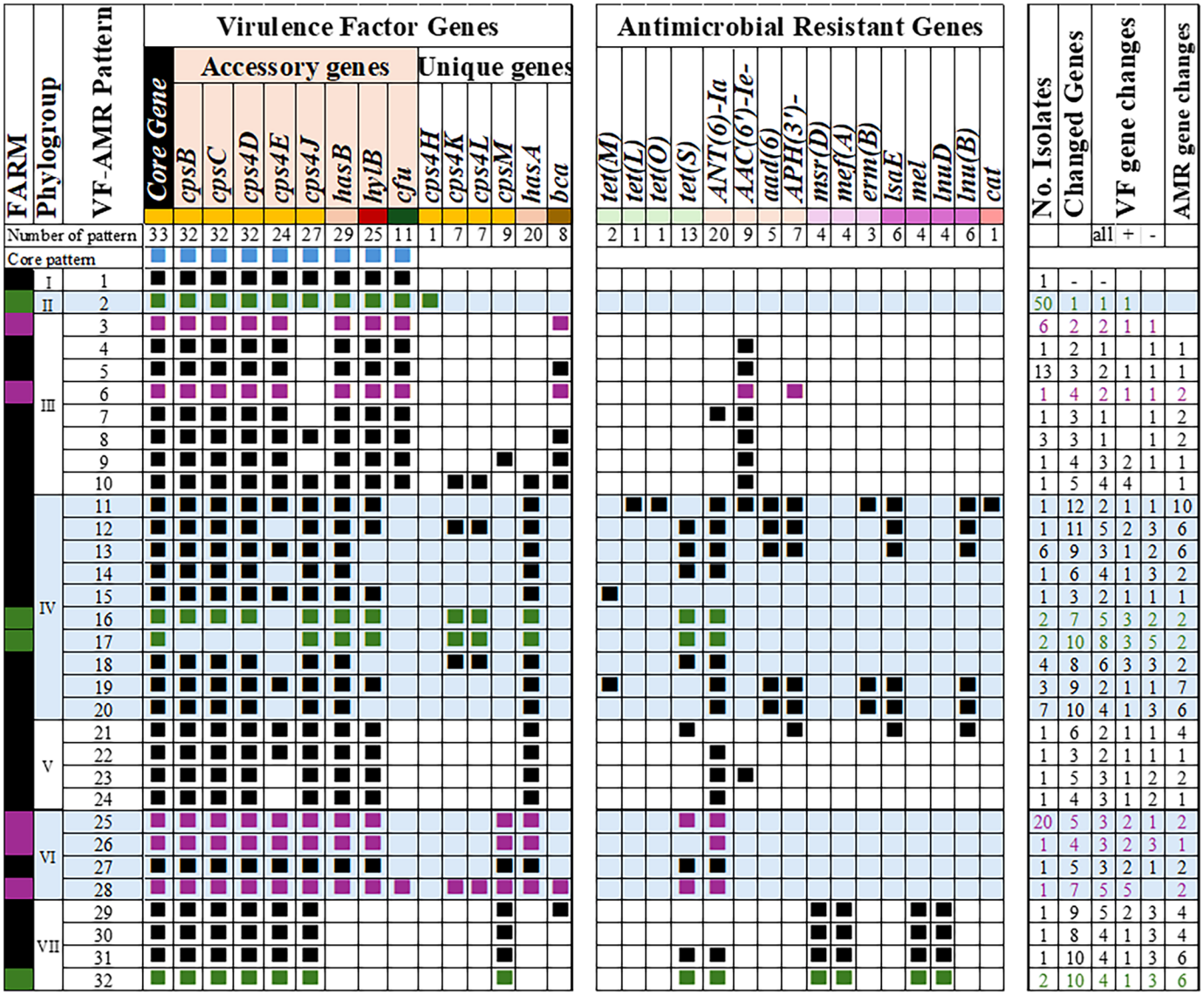



**Figure 2.** Patterns of virulence factor (VF) and antimicrobial resistance (AMR) genes in *Streptococcus uberis* isolates from bovine mastitis cases, illustrating diversions from the core gene pattern. The first 3 columns represent farms, phylogenetic groups, and pattern ID, respectively. The left matrix displays core genes found in all isolates, with the number of accessory genes indicating isolates lacking these genes, and the number of genes in unique groups showing which isolates possess them. The core pattern consists of both core and accessory virulence genes. The middle matrix showed the distribution of AMR genes across each pattern, and the right matrix summarizes the total number of isolates per pattern and the number of gene deviations from the core pattern for both VF and AMR gene.

including *tet(S)*, *hasA*, *bca*, *ant(6)-Ia*, and *AAC(6')-Ie-APH(2")-Ia* ($P < 0.01$). In terms of the relationship between VF and AMR genes, the 2 most frequent AMR genes showed strong associations. *tet(S)* was significantly associated with *cfu*, *cps4K/cps4L*, and *hasA* ($P < 0.05$), whereas *ant(6)-Ia* associated with the presence of *cfu* and *hasA*, and the absence of *bca* ($P < 0.01$). Overall, the AMR genes were significantly associated with the presence of *cfu* ($P < 0.05$). These findings suggest a complex interplay between specific VF and AMR in *Strep. uberis* isolates.

## DISCUSSION

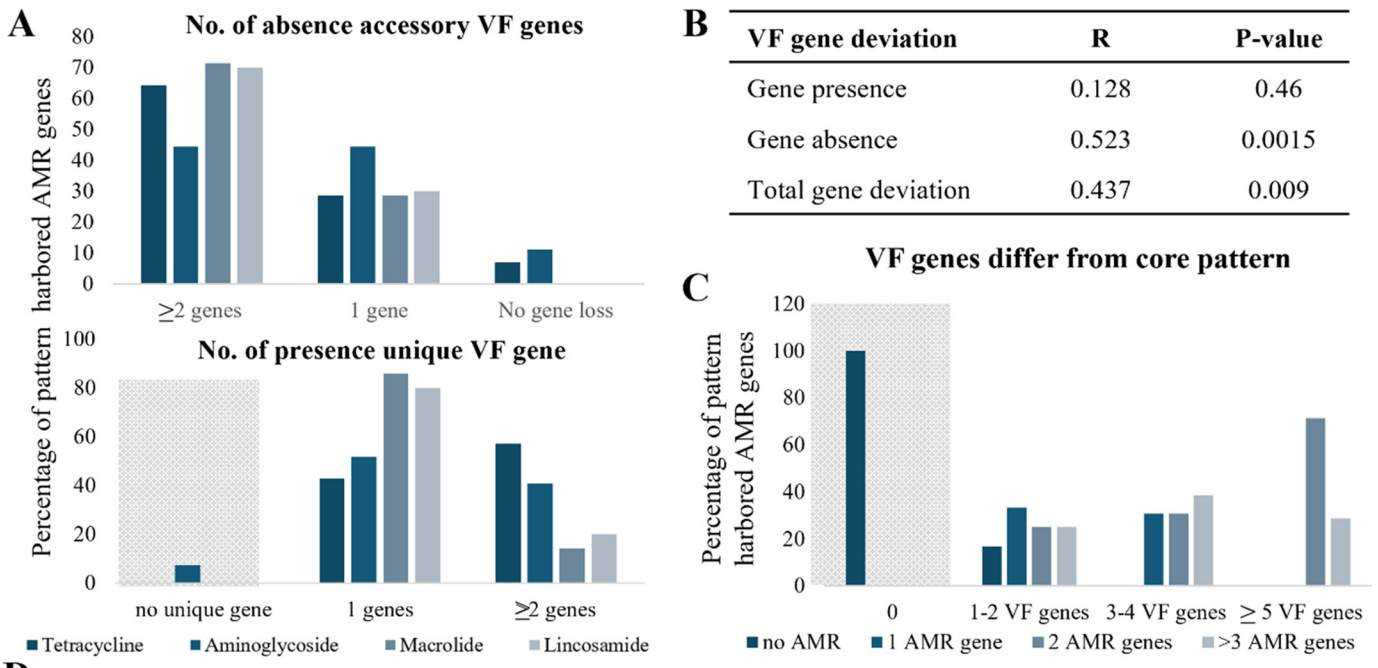
This study aimed to investigate the genetic variations in presence or absence of VF and AMR genes in *Strep. uberis* and to explore the potential contributing management factors in 3 farms with a high prevalence of *Strep. uberis* mastitis. Furthermore, unregulated antimicrobial use without veterinary oversight likely contributed to the high levels of AMR, which may potentially reduce mastitis cure rates on these farms (Abdi et al., 2018). In particular, poor milk quality management practices in

**Table 2.** Percentage of patterns with specific identified genes or gene groups and their associations with phylogenetic groups (I to VII) and farms (A, B, and C)

| | Phylogroup | | | | | | | | Farm | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene | I | II | III | IV | V | VI | VII | *P*-value | A | B | C | *P*-value |
| Number of patterns | 1 | 1 | 8 | 10 | 4 | 4 | 4 | | 5 | 23 | 5 | |
| VF genes | | | | | | | | | | | | |
| *cps4E* | 100 | 100 | 100 | 40 | 50 | 100 | 100 | <0.01 | 0 | 26 | 60 | 0.15 |
| *cps4J* | 100 | 100 | 25 | 100 | 100 | 100 | 100 | <0.01 | 40 | 17 | 0 | 0.41 |
| *hylB* | 100 | 100 | 100 | 60 | 100 | 100 | 0 | <0.01 | 0 | 30 | 20 | 0.58 |
| *cfu* | 100 | 100 | 100 | 0 | 0 | 25 | 0 | <0.01 | 40 | 70 | 80 | 0.51 |
| *cps4H* | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0.06 | 0 | 0 | 20 | 0.3 |
| *cps4K/cps4L* | 0 | 0 | 13 | 40 | 0 | 25 | 0 | 0.43 | 20 | 13 | 60 | 0.1 |
| *cpsM* | 0 | 0 | 13 | 0 | 0 | 100 | 100 | <0.01 | 60 | 22 | 20 | 0.24 |
| *hasA* | 0 | 0 | 13 | 100 | 100 | 100 | 0 | <0.01 | 60 | 61 | 60 | 1 |
| *bca* | 0 | 0 | 75 | 0 | 0 | 25 | 25 | <0.01 | 60 | 22 | 0 | 0.09 |
| Virulence groups | | | | | | | | | | | | |
| Exopolysaccharide | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 1 | | | | |
| Capsule formation | 100 | 100 | 88 | 0 | 0 | 0 | 100 | <0.01 | 40 | 39 | 40 | 0.68 |
| Cytolysis | 0 | 0 | 0 | 36 | 0 | 0 | 100 | <0.01 | 0 | 30 | 20 | 0.47 |
| Hemolysin | 0 | 0 | 0 | 100 | 100 | 75 | 100 | <0.01 | 40 | 70 | 80 | 0.27 |
| Surface-associated proteins | 100 | 100 | 25 | 100 | 100 | 75 | 75 | 0.015 | 40 | 78 | 100 | 0.13 |
| AMR genes | | | | | | | | | | | | |
| *tets* | 0 | 0 | 0 | 60 | 25 | 75 | 50 | 0.02 | 40 | 30 | 80 | 0.2 |
| *ANT* | 0 | 0 | 13 | 90 | 75 | 100 | 50 | <0.01 | 60 | 57 | 80 | 0.86 |
| *AAC* | 0 | 0 | 88 | 9.1 | 25 | 0 | 0 | <0.01 | 20 | 35 | 0 | 0.49 |
| *aad* | 0 | 0 | 0 | 50 | 0 | 0 | 0 | 0.12 | 0 | 22 | 0 | 0.44 |
| *APH* | 0 | 0 | 13 | 50 | 25 | 0 | 0 | 0.43 | 20 | 26 | 0 | 0.8 |
| *erm* | 0 | 0 | 0 | 30 | 0 | 0 | 0 | 0.45 | 0 | 13 | 0 | 1 |
| *lseE/lnub* | 0 | 0 | 0 | 50 | 25 | 0 | 0 | 0.17 | 0 | 26 | 0 | 0.5 |
| AMR classes | | | | | | | | | | | | |
| Tetracycline | 100 | 100 | 100 | 9.1 | 75 | 25 | 50 | <0.01 | 60 | 57 | 20 | 0.34 |
| Aminoglycoside | 100 | 100 | 13 | 9.1 | 0 | 0 | 50 | 0.01 | 20 | 17 | 20 | 0.26 |
| Macrolide | 100 | 100 | 100 | 73 | 100 | 100 | 0 | <0.01 | 15 | 74 | 80 | 0.57 |
| Lincomycin | 100 | 100 | 100 | 55 | 75 | 100 | 0 | 0.01 | 100 | 61 | 80 | 0.28 |
| AMR | 0 | 0 | 88 | 37 | 13 | 13 | 13 | 0.01 | 80 | 96 | 80 | 0.21 |

these farms contributed to high rates of IMI, leading to the continuous predominance of *Strep. uberis* IMI. The primary selection of representative strains based solely on the duration of the presence of the same species may have influenced the observed genetic diversity. Given the limited number of farms, the nonrandom sampling of herds, and the clustering of isolates within both farms and phylogroups, our findings should be interpreted with caution. Fisher's exact tests with FDR correction were used to address multiple comparisons (Richards et al., 2011) and remain a valid approach for detecting associations in small sample sizes. However, to fully capture the hierarchical structure of the data, future studies may benefit from using additional statistical tools, such as Scoary, which incorporates post hoc label-switching permutation tests to further reduce the likelihood of false positives (see for example, Brynildsrud et al., 2016 and Greening et al., 2021). Moreover, integrating genome-wide comparisons with publicly available *Strep. uberis* genomes could help place our findings in a broader evolutionary and epidemiological context.

Three categories of *Strep. uberis* VF genes were determined in this study. As shown in Figure 1, 21 core VF genes were found in all isolates, and these were the highly conserved genes that may be crucial for survival, pathogenicity exhibition, and adaptation across various hosts and environments (Perrig et al., 2015; Calonzi et al., 2020; Zouharova et al., 2022b). These core genes, including biofilm putative glycosyltransferase, *hasC*, *lmb*, *sclB*, *sua*, *fbpS*, *srtA*, *fbp54*, and others, were also reported by Vezina et al. (2021). In contrast, accessory and unique VF genes exhibited strain-specific patterns that varied across isolates. Results showed the genetic diversity of *Strep. uberis* strains and the distinct environment in which they evolve. This finding might be explained by the theory presented by Shi et al. (2022), which states that bacteria adapt through mutation and selection. Bacteria undergo adaptive evolution when the selection pressure is strong and persistent (Horinouchi and Furusawa, 2020). Genetic adaptation in mastitis-causing bacteria also involves horizontal gene transfer (Schönknecht et al., 2014; Vezina et al., 2021), co-evolution of bacteria in the udder environment (Hoque et




**B**

| VF gene deviation | R | P-value |
|---|---|---|
| Gene presence | 0.128 | 0.46 |
| Gene absence | 0.523 | 0.0015 |
| Total gene deviation | 0.437 | 0.009 |

**D**

| | *cps4E* w/o-with | *cps4J* w/o-with | *hylb* w/o-with | *cfu* w/o-with | *cps4K/cps4L* w/o-with | *cpsM* w/o-with | *hasA* w/o-with | *bca* w/o-with | *tetS* w/o-with | *ANT* w/o-with | *AAC* w/o-with | *aad(6)* w/o-with | *APH* w/o-with | *erm(B)* w/o-with |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Numbers | 24-9 | 27-6 | 25-8 | 11-22 | 26-7 | 24-9 | 13-20 | 25-8 | 20-13 | 13-20 | 24-9 | 28-5 | 26-7 | 30-3 |
| **%gene deviation** | | | | | | | | | | | | | | |
| **Virulence genes** | | | | | | | | | | | | | | |
| *cps4J* | 25-0 | | | | | | | | | | | | | |
| *hy1B* | 20-33 | 30-0 | | | | | | | | | | | | |
| *cfu* | 24-100 | 81-0 ** | 56-100 | | | | | | | | | | | |
| *cps4K/cps4L* | 8-56 | 26-0 | 24-13 | 18-23 | | | | | | | | | | |
| *cpsM* | 38-0 | 30-17 | 20-50 | 18-32 | 31-14 | | | | | | | | | |
| *hasA* | 46-100 | 74-0 * | 64-50 | 18-82 ** | 50-100 * | 67-44 | | | | | | | | |
| *bca* | 33-0 | 15-67 * | 28-13 | 64-5 ** | 23-29 | 21-33 | 46-10 | | | | | | | |
| **AMR genes** | | | | | | | | | | | | | | |
| *Tet(S)* | 29-67 | 48-0 | 32-63 | 9-56 * | 27-86 * | 33-56 | 15-55 * | 48-13 | | | | | | |
| *ANT(6)-Ia* | 46-100 | 70-17 | 56-75 | 18-82 ** | 54-86 | 58-67 | 23-85 ** | 76-13 ** | 40-92 ** | | | | | |
| *AAC(6')-Ie-APH(2")-Ia* | 33-11 | 15-83 ** | 36-0 | 63-9 ** | 31-14 | 33-11 | 46-15 | 16-63 * | 45-0 * | 46-15 | | | | |
| *aad(6)* | 13-22 | 19-0 | 12-25 | 0-23 | 15-14 | 21-0 | 0-25 | 20-0 | 15-15 | 0-25 * | 17-11 | | | |
| *APH(3')-IIIa* | 21-22 | 22-17 | 20-25 | 9-27 | 23-14 | 29-0 | 7-30 | 24-13 | 20-23 | 15-25 | 21-22 | 6-100 ** | | |
| *erm(B)* | 8-11 | 11-0 | 8-13 | 0-13 | 11-0 | 13-0 | 0-15 | 12-0 | 15-0 | 0-15 | 8-11 | 0-60 ** | 0-43 ** | |
| *lsaE/lnub* | 17-22 | 22-0 | 16-25 | 0-27 | 19-14 | 25-0 | 0-30 * | 24-0 | 15-23 | 8-25 | 21-11 | 4-100 ** | 0-86 ** | 10-100 ** |
| AMR genes | 88-100 | 93-83 | 88-100 | 73-100 * | 88-100 | 88-100 | 77-100 | 92-88 | - | - | - | - | - | - |

**Figure 3.** Relationship between virulence factor (VF) genes and antimicrobial resistance (AMR) genes. (A) The percentages of patterns harboring AMR genes varied among VF genes deviating from the core pattern. Statistical significance at the pattern level (n = 32, $P < 0.05$) was determined using Fisher's exact test. (B) The correlation between gene presence or absence in antimicrobial resistance and VF genes, analyzed using the Spearman's rank correlation. (C) The percentages of patterns harboring AMR genes varied based on the number of accessory (top) and unique (bottom) VF genes. The gray area refers to a group without any genes differ from a core pattern. (D) The percentages of gene deviations represent the proportion of deviations by gene from the row compared with the gene in the column, analyzed using the Fisher's exact test. The false discovery rate procedure of Benjamini and Hochberg (1995) was used. *$P < 0.05$, **$P < 0.01$.

al., 2020), specific gene mutations (Szyda et al., 2019), or genes transferred through plasmids or phages (Vezina et al., 2022). Although the exact mechanisms driving the genetic adaptation and diversity of *Strep. uberis* in different environments remain unclear, this study highlights potential factors influencing gene acquisition, loss, and adaptation in these settings.

Several potential factors might contribute to the large genetic diversity of *Strep. uberis*. This study reported a clear association between phylogroups and farms. As shown in Table 2, most variations in VF and AMR genes, along with their respective groups or classes, were significantly associated with specific phylogroups and farms, suggesting that these lineages may be adapting

to distinct ecological niches, farm conditions, or other selective pressures. These findings align with the study by Woudstra et al. (2023), which suggested that the prevalence and duration of *Strep. uberis* infections are influenced by individual farm management practices and cow characteristics. The low diversity of *Strep. uberis* in farms A and C might indicate transmission through contagious routes, whereas the high diversity in farm B suggested unconnected infection events (Zadoks and Schukken, 2006). This finding is consistent with Zouharova et al. (2022a), indicating that they detected multiple genotypes within a single herd. Meanwhile, the findings of Zadoks et al. (2003) and Fenske et al. (2022) reported a few dominant *Strep. uberis* strains in herds suggesting mainly contagious transmission. At the farm level, several studies have demonstrated low diversity in contagious bacteria (Haveri et al., 2008; Woudstra et al., 2023), though environmental hotspots cannot be entirely ruled out. Additionally, our results found the proportion of isolates harboring at least one AMR gene was 79.3% (23/29) in farm A, 98.1% (52/53) in farm B, and 10.7% (6/56) in farm C. Notably, a high number of strains (81.1%, 112/138) carried plasmids, with ~32% of them linked to AMR genes, higher than reported in previous studies (Thomas et al., 2024). This may be attributed to the high antimicrobial use on these farms, which could have influenced the observed genetic diversity. The lower AMR detected genes in farm C were consistent across isolates with the same gene pattern, suggesting the virulence and resistance genes might transferred through horizontal gene transfer (von Wintersdorff et al., 2016). Meanwhile, the higher resistance rates in farms A and B might be associated with poorly regulated access to antibiotics and high antibiotic exposure of bacterial isolates as a result. Sharma et al. (2020) reported that limited access to veterinarians and high treatment costs led farmers to rely on past prescriptions and self-administer antibiotics instead of seeking professional consultation. In addition, the variation in VF and AMR genes, particularly the absence of AMR genes in farm C, may reflect true biological differences or could result from sequencing or assembly limitations, such as incomplete genomes or contig gaps. Future analyses incorporating more detailed assembly metrics and gap assessments would help verify the true absence of these genes more reliably.

Previous studies have shown a connection between VF and AMR. Sometimes, virulence and resistance may evolve at different times; however, they are not independent traits. Instead, their relationship depends on various factors, including the bacterial species, the mechanisms of resistance and virulence, the ecological niche, environmental conditions, and the host's immune system status (Cepas and Soto, 2020). A significant negative relationship was found between the extent of genetic variation and the patterns harboring AMR genes in this study (Figure 3). Although antibiotic resistance and bacterial virulence have evolved over different timescales, there is likely an interplay, or coselection, between antibiotic-resistant genes and VF under selective pressure (Geisinger and Isberg, 2017; Cepas and Soto, 2020; Pan et al., 2020; Zou et al., 2023). Our results on the acquisition of resistance, accompanied by the loss of virulence genes, were consistent with those of previous studies. For example, a negative correlation was found in *E. coli* (Pompilio et al., 2018; Ballén et al., 2022; LaMontagne et al., 2023) and streptococci isolates from cows (Ding et al., 2023). For uropathogenic strains of *E. coli*, the acquisition of antibiotic resistance can induce the loss of VF and biofilm formation; in other cases, an increase in antibiotic resistance enhances virulence capacity (Cepas and Soto, 2020). These adjustments might enable bacteria to survive in environments with antibiotics by allocating sufficient energy and resources toward maintaining resistance mechanisms rather than VFs (Beceiro et al., 2013; Melnyk et al., 2015). Additionally, mechanisms that are involved in the dissemination of virulence and resistance are, for example, the presence of prophages (Cepas and Soto, 2020) or MGE carrying both gene groups (Bunduki et al., 2021).

In addition, there were significant (positive and negative) correlations between certain resistant genes and specific VF genes, especially *cfu* and *hasA* (Figure 3D). Previous research has reported similar connections, noting that hemolysins indirectly increase bacterial resistance to antibiotics (Motamedi et al., 2018; Cepas and Soto, 2020), and that reduced capsule density also enhances antibiotic evasion (D'Angelo et al., 2023). The relationship between AMR and virulence has been described in the literature by Beceiro et al. (2013); both are critical for bacterial survival under adverse conditions, allowing pathogens to evade host defenses, resist antimicrobial treatments, and adapt to new competitive environments. These scenarios might be affected by other factors such as environmental conditions, the mechanism of resistance of bacteria, and hosts' immune efficiency.

Genes encoding the formation of capsular polysaccharides, *cpsB*, *cpsC*, *and cpsD*, were found in all isolates. This implies that all isolates had the potential of forming a capsule and, in this way, become more resilient to host immune mechanisms, such as immunoglobulins or complement. Recent investigations on *Strep. uberis* isolates from transient and persistent IMI showed that all isolates from persistent intramammary infections carried both *hasA* and *hasB*, whereas the majority of isolates from transient infections did not (Srithanasuwan et al., 2022). This suggests that the ability of capsule formation is essential for persistence in the mammary gland of the host.

## CONCLUSIONS

This study demonstrates the genetic diversity, virulence, and AMR of *Streptococcus uberis* causing mastitis in farms with poor mastitis control and high antibiotic use. The findings suggest that bacterial infection characteristics and farm management practices, particularly antimicrobial use, may influence this diversity and gene variation. Antimicrobial resistance prevalence was strongly linked to the policy of antibiotic usage and drug regulation specific to each farm. Furthermore, a negative correlation between virulence and AMR was observed, providing an example of the bacteria's ability to adapt and thrive under varying environmental pressures in mastitis conditions. Given the significant effect of *Strep. uberis* on bovine mastitis, further research involving a larger number of farms is essential to better understand how farm management practices and the interplay between virulence and resistance genes influence IMI in mastitis-prevalent herds.

## NOTES

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**Nonstandard abbreviations used:** AMR = antimicrobial resistance, FDR = false discovery rate; MGE = mobile genetic elements; VF = virulence factor; WGS = whole-genome sequencing.

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