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Pathogenic and symbiotic bacteria in ruminants: Antimicrobial resistance and microbial homeostasis

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Pathogenic and symbiotic bacteria in ruminants: Antimicrobial resistance and microbial homeostasis

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Editorial: Pathogenic and symbiotic bacteria in ruminants: antimicrobial resistance and microbial homeostasis

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Editorial on the Research Topic
Pathogenic and symbiotic bacteria in ruminants: antimicrobial resistance and microbial homeostasis

Ruminants share a profound connection with human beings. They have the remarkable ability to harness rumen microorganisms, facilitating the fermentation process and converting plant proteins into high-quality animal proteins. This process yields a significant supply of meat and dairy products indispensable to our lives. The diversity and abundance of rumen microorganisms indicate not only the host's digestive and metabolic capacity but also its health status. During the long process of evolution, ruminants have adapted to environmental changes by modifying the types and abundance of microorganisms in their rumen, thereby optimizing energy utilization (Yang et al.).

Nonetheless, alongside the beneficial symbiotic bacteria in the rumen, numerous pathogenic bacteria pose a grave threat to the wellbeing of ruminant animals. Brucellosis is a zoonosis of significant public health and economic importance that is endemic in ruminants worldwide. Compared with large farmed ruminants (cattle, zebu, and buffalo), small farmed ruminants (goats and sheep) infected with *Brucella melitensis* or *B. ovis* pose a greater threat to humans, especially in African countries (Hussen et al.).

The dairy market primarily comprises milk from cows, goats, water buffaloes, and camels, with cow's milk taking the lead in consumption. However, goat, water buffalo, and camel milk stand out due to their distinctive nutritional components, making them particularly suitable for specific demographic groups. Mastitis is the most common, costly, and important disease in the dairy industry. Up to now, more than 150 species of pathogenic bacteria have been identified in the raw milk of animals with mastitis. *Staphylococcus aureus* is frequently isolated in many countries (1). For example, Wang K. et al. have identified a high pooled prevalence of *S. aureus* (36.23%) in China from 2000 to 2020. Although *S. aureus* has been well documented and recognized as a significant mastitis-causing organism in cows, its molecular characteristics and pathogenicity in water buffaloes are largely unknown. A recent epidemiological study conducted in Guangdong province, China, revealed that

S. aureus is the third most prevalent pathogenic bacteria in water buffaloes with subclinical mastitis, and its isolation frequency is lower than *Escherichia coli* and coagulase-negative staphylococci (Zhang et al.).

Due to the frequent occurrence of mastitis and repeated use of antibiotics (administered during the lactation, or at dry-off) (Okello et al.), bacteria isolated from raw milk are gradually developing antimicrobial resistance. In recent years, the antibiotic resistance phenotypes and genotypes of mastitis pathogens, as well as the development of new antibiotic replacement therapies, have become prominent areas of study. For example, Toquet et al. has reported the *in vivo* antimicrobial potential of lactic acid bacteria (a kind of probiotics) in the treatment of contagious agalactia caused by *Mycoplasma agalactiae*. Although hundreds of pathogenic bacteria are associated with mastitis, studies on antibiotic resistance are mainly focused on *S. aureus*, *Escherichia coli* and *Streptococcus*. A high prevalence of *S. aureus* has been reported to be resistant to penicillin G, ampicillin, or amoxicillin. In contrast, a low prevalence of isolates was resistant to trimethoprim-sulfamethoxazole or gentamycin (2). *E. coli* and *Streptococcus* are also major mastitis-causing pathogens in dairy cows. Multidrug-resistant (acquired resistance to $\geq$ three classes of antimicrobials) *Streptococcus* can be frequently isolated from raw milk of cows with mastitis, with the presence of antibiotic resistance genes and virulence genes (3, 4).

In addition to mastitis, veterinarians should pay much attention to respiratory and digestive diseases caused by pathogenic bacteria infection. Diarrhea can be caused by different kinds of pathogens, including bacteria (*E. coli* K99/ O157 and *Salmonella enteritidis*), viruses (bovine viral diarrhea virus, bovine and ovine rotavirus), and parasites (*Cryptosporidium* sp. and *Coccidium* sp.) (Wang D. et al.). Respiratory disease can result in slow weight gain in beef cattle and sheep, causing considerable financial losses for beef and lamb producers. Airway microbiotas enriched with probiotics (such as *Lactobacillus*) are associated with good respiratory health. On the contrary, microbiotas enriched with recognized pathogenic bacteria (*Klebsiella pneumoniae* and *Pasteurella multocida*) are related to respiratory diseases (5). The threat of respiratory and digestive disease for cattle, sheep and goat operations is exacerbated by increasing prevalence of antimicrobial resistance in pathogenic bacteria (Carter et al.).

Anaplasma, a kind of tickborne pathogen, can cause anaplasmosis in ruminants. Previous studies have demonstrated the presence of *A. marginale*, *A. ovis*, *A. platys*, and *A. phagocytophilum* in ruminants, and *A. marginale* (Mahmoud et al.) and *A. phagocytophilum* can be frequently detected (6, 7). It is worth noting that *A. phagocytophilum* is a zoonotic pathogen that can cause human granulocytic anaplasmosis (HGA). HGA is characterized by sustained high fever and a decrease in white blood cells and platelets. Its clinical manifestations primarily include overall discomfort, fatigue, headache, muscle soreness, and symptoms like nausea, vomiting, loss of appetite, and diarrhea. Misdiagnosis is common due to the similarity of its symptoms to certain viral infectious diseases. In severe cases, it can lead to multiple organ dysfunction, including the heart, liver, and kidneys, potentially resulting in fatal outcomes.

In conclusion, the contributions to this Research Topic expand our understanding of the distributions and characteristics of pathogenic and symbiotic bacteria in ruminants, providing valuable insights to improve our ability to safeguard the health of these animals.

Author contributions

YY: Writing – original draft, Writing – review & editing. JZ: Writing – review & editing. HE-M: Writing – review & editing.

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Conflict of interest

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BRIEF RESEARCH REPORT
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Assessment of Diversity of Antimicrobial Resistance Phenotypes and Genotypes of *Mannheimia haemolytica* Isolates From Bovine Nasopharyngeal Swabs

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The threat of bovine respiratory disease (BRD) for cattle operations is exacerbated by increasing prevalence of antimicrobial resistance (AMR) in *Mannheimia haemolytica*, a leading cause of BRD. Characterization of AMR in *M. haemolytica* by culture and susceptibility testing is complicated by uncertainty regarding the number of colonies that must be selected to accurately characterize AMR phenotypes (antibiograms) and genotypes in a culture. The study objective was to assess phenotypic and genotypic diversity of *M. haemolytica* isolates on nasopharyngeal swabs (NPS) from 28 cattle at risk for BRD or with BRD. NPS were swabbed onto five consecutive blood agar plates; after incubation up to 20 *M. haemolytica* colonies were selected per plate (up to 100 colonies per NPS). Phenotype was determined by measuring minimum inhibitory concentrations (MIC) for 11 antimicrobials and classifying isolates as resistant or not. Genotype was indirectly determined by matrix-assisted laser desorption/ionization time of flight mass spectroscopy (MALDI-TOF MS). NPS from 11 of 28 cattle yielded at least one *M. haemolytica* isolate; median (range) of isolates per NPS was 48 (1–94). NPS from seven cattle yielded one phenotype, 3 NPS yielded two, and 1 NPS yielded three; however, within a sample all phenotypic differences were due to only one MIC dilution. On each NPS all *M. haemolytica* isolated were the same genotype; genotype 1 was isolated from three NPS and genotype two was isolated from eight. Diversity of *M. haemolytica* on bovine NPS was limited, suggesting that selection of few colonies might adequately identify relevant phenotypes and genotypes.

Keywords: cattle, respiratory, bacteria, pasteurellaceae, resistance

INTRODUCTION

Bovine respiratory disease (BRD), the leading cause of morbidity and mortality in U.S. beef cattle (1), poses a threat to cattle operations. The prevalence of antimicrobial resistance (AMR) appears to be increasing in *Mannheimia haemolytica* (*M. haemolytica*), a leading contributor to BRD (2–4); research is underway to determine the causes and impact of AMR in *M. haemolytica*. Characterization of the AMR phenotype by culture and antimicrobial susceptibility testing is complicated by uncertainty regarding the number of *M. haemolytica* colonies that must be selected to adequately characterize antimicrobial susceptibility phenotypes (antibiograms) and genotypes in a sample. While multiple colonies consistent with *M. haemolytica* may be present on a primary culture plate from a bovine sample, standard diagnostic methodology is to select one isolate for characterization. It may be that selection of multiple colonies is necessary to accurately identify important AMR isolates, but this could substantially amplify the cost of testing. As research is ongoing to characterize the extent and impact of AMR in the bacteria that contribute to BRD, it is important to clarify whether a single isolate from a respiratory sample adequately represents the characteristics of all isolates that can be identified in the same sample.

The number of colonies that must be isolated from a primary culture plate to accurately represent the diversity of isolates on the plate has been determined for other bacteria (5, 6), but to our knowledge this number has not been estimated for *M. haemolytica*. The study objective was to describe the phenotypic and genotypic diversity of up to 100 *M. haemolytica* isolates from individual bovine nasopharyngeal swabs (NPS) collected from live cattle at risk for BRD, or after treatment for BRD.

MATERIALS AND METHODS

Animals

Subject cattle ($n = 28$) were a convenience sample of post-weaned mixed breed beef cattle of *Bos taurus* origin, weighing 180–270 kg, with an estimated age of 6 months to 1 year. The cattle were in different groups of recently purchased and comingled cattle from various auction markets. At the time of sampling the cattle had received zero to three treatments with an antimicrobial approved for treatment of BRD. The cattle were sampled either at a convenient time post arrival or when they were removed from their pen to be treated for BRD. Because the primary objective of the study was to describe the variability of *M. haemolytica* phenotypes and genotypes isolated from bovine NPS, a mix of both previously treated and untreated cattle was included so that it was possible to ascertain whether previous treatment was likely to impact variability. Sample collection for this study was approved by the Mississippi State University Institutional Animal Care and Use Committee (IACUC 17-330).

Nasopharyngeal Swab (NPS) Collection and Culture

Double guarded swabs (#022964 MWI, Nampa, ID, USA) were used to sample the nasopharynx of cattle as previously described (7). One swab was collected from each nostril then the two swabs were placed together into transport media (Modified Amies Clear gel, SP130X, Starplex Scientific Inc. Etobicoke, Ontario, Canada) and transported back to the laboratory on ice for culture within 6 h of collection. Both swabs were streaked together on the first quadrant of 5 sequential plates containing tryptic soy agar + 5% sheep's blood (blood agar) plates. Five sequential plates were streaked in order to account for the possibility that overgrowth of contaminant bacteria might prevent identification of *M. haemolytica* on the first plate. For each of the 5 plates a new sterile loop was used to streak from the first quadrant to the remaining 3 quadrants. Plates were streaked and evaluated in a biosafety cabinet to prevent contamination. After streaking, plates were incubated at 37°C, 5% CO_2 for 18–24 h, then colonies phenotypically consistent with *M. haemolytica* (round white/gray with a glossy edge and beta hemolysis) were collected, with one colony tested by the oxidase (slow +), indole (–), catalase (+), and KoH (+) tests to confirm identity. If there were fewer than 3 colonies on a plate the biochemical tests were not performed until the isolates on the primary plate were subcultured. Isolated colonies consistent with *M. haemolytica* were subcultured to a new plate; a maximum of 20 colonies were collected from each of the 5 plates, for up to 100 colonies per NPS. The subcultures were incubated at 37°C in 5% CO_2 for 18–24 h, then for each subculture plate all bacteria were swabbed off and transferred to 1 ml of 50% glycerol in 1X phosphate buffered saline, and stored at −80°C.

Broth Microdilution for Determination of Antimicrobial Susceptibility

Twelve to 14 months after the NPS were collected, isolates were removed from −80°C storage, transferred to ice, and immediately streaked onto blood agar plates. After 18–24 h of incubation at 37°C in 5% CO_2 each isolate was tested to confirm genus and species using an automated system (Sensititre, ThermoFisher etc., plate YGNID), and the minimum inhibitory concentration (MIC) for 11 antimicrobials was determined by broth microdilution (Sensititre, ThermoFisher etc., plate YBOPO7F) at the Mississippi State University College of Veterinary Medicine Diagnostic Laboratory (MSU CVM DL). MICs were determined for ceftiofur, danofloxacin, enrofloxacin, florfenicol, gamithromycin, penicillin, spectinomycin, tetracycline, tildipirosin, tilmicosin, and tulathromycin; each isolate was identified as susceptible, intermediate, or resistant based on CLSI-defined breakpoints for *M. haemolytica* in bovine respiratory disease. For subsequent evaluation in this study, intermediate isolates were grouped with susceptible isolates, so that each isolate was defined as resistant or non-resistant. A figure representing the phenotypes represented by isolates (**Figure 1**) was constructed in R v4.0.4, using the Bioconductor package ComplexHeatmap v2.10.0 (8). Color scaling was performed with the R package viridis v0.6.2 (9) to allow ease of visual interpretation for individuals with color blindness.

MALDI-TOF MS

For each isolate, the broth used for MIC determination was also used to inoculate a blood agar plate which was incubated

for 18–24 h at 37°C in 5% CO_2 for confirmation of identity by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). If a plate inoculated with broth used for antimicrobial susceptibility testing yielded no growth or contaminated growth, so that the isolate could not also be confirmed as *M. haemolytica* by MALDI-TOF MS, that isolate was omitted from the analysis. This led to exclusion of 9 of 57 isolates from animal 205, 10 of 51 isolates from animal 260, 3 of 73 isolates from animal 277, and 2 of 61 isolates from animal 256. For MALDI-TOF MS isolates were shipped by overnight mail to the University of Nebraska-Lincoln Veterinary Diagnostic Center (UNL VDC), where isolated colonies were prepared in duplicate according to manufacturer's recommended procedures for the direct smear method using a α-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics, Billerica, MA, USA) and subjected to automatic detection in positive linear mode between 2 kDA and 20 kDA m/z, with a laser frequency of 60 Hz using a Microflex LT MALDI-TOF mass spectrometer (Bruker Daltonics) calibrated for reference masses of 3,637–16,952 Da using the manufacturer's supplied bacterial test standard. Identifications were determined using commercial software (Bruker Biotyper, Bruker Daltonics) and the manufacturer's database (BDAL v10 containing 9,607 reference spectra) that has been supplemented with an in-house developed library with additional *Mannheimia* spp. reference spectra. Isolates were identified to the species level if match scores on at least one replicate were ≥ 2.2. The MALDI-TOF MS profile generated during identification was also used to assign each isolate to genotype 1 or 2 as previously described using Clinprotools 3.0 software (Bruker Daltonics) with quick classifier model, which was developed based on whole genome sequences of known genotype 1 and genotype 2 *M. haemolytica* isolates, of which there are >26,000 nucleotide polymorphisms that discriminate between the two genotypes. In addition to the classifier model, a manual review of raw mass spectrum

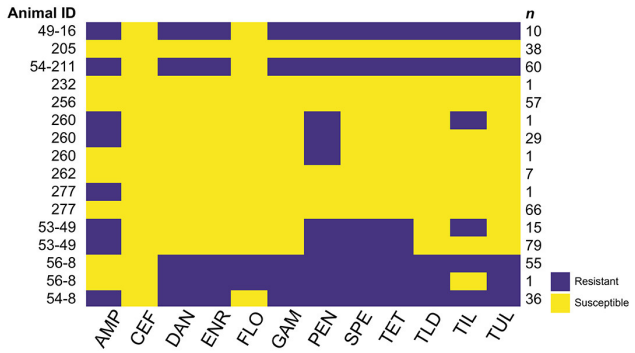

FIGURE 1 | Antimicrobial resistance (AMR) phenotypes (antibiograms) of *M. haemolytica* isolates from nasopharyngeal swabs (NSP) collected from 11 cattle. The median number (range) of *M. haemolytica* isolates obtained from each animal was 48 (1–94). Antimicrobials: ampicillin (AMP), ceftiofur (CEF), danofloxacin (DAN), enrofloxacin (ENR), florfenicol (FLO), gamithromycin (GAM), penicillin (PEN), spectinomycin (SPE), tetracycline (TET), tildipirosin (TLD), tilmicosin (TIL), and tulathromycin (TUL). Yellow cells indicate that the isolates were not resistant to the antimicrobial indicated, while purple cells indicate resistance. While multiple AMR phenotypes were identified among *M. haemolytica* isolates from four cattle (260, 277, 53–49, and 56–8), the difference in phenotype was in all cases due to a difference of only a single dilution in the broth microdilution assay, which led to the isolate changing from not resistant to resistant for only one or two antimicrobials. The relevant minimum inhibitory concentration (MIC) data are presented in **Supplementary Material 1**.

TABLE 1 | Information regarding cattle from which *Mannheimia haemolytica* (*M. haemolytica*) was isolated from nasopharyngeal swabs (NPS), the number of AMR phenotypes (antibiograms) of M. haemolytica identified among all isolates, and the genotype of *M. haemolytica* isolated based on MALDI-TOF MS.

Animal ID	Housing group	Clinical signs of BRD when sampled	Antimicrobials received before sampling (days before)	Number of AMR phenotypes	Genotype
205	A	no	none	1	1
232	A	no	none	1	1
260	A	no	none	3	2
277	A	no	none	2	2
256	A	no	none	1	1
262	A	no	none	1	2
56–8	B	yes	CEF (34) TUL (19) FLO (9)	2	2
53–49	C	yes	CEF (8)	2	2
49–16	D	yes	CEF (35) TUL (15) FLO (12)	1	2
54–8	D	yes	CEF (21) TUL (3)	1	2
54–211	D	yes	CEF (14) TUL (7)	1	2

Cattle in the same housing group were housed and managed together. Although multiple phenotypes of M. haemolytica were isolated from some cattle, all isolates identified from each animal were a single genotype, either genotype 1 or genotype 2. Antimicrobials: CEF, ceftiofur, TUL, tulathromycin, FLO, florfenicol.

TABLE 2 | Number of *Mannheimia haemolytica* (*M. haemolytica*) isolates recovered from nasopharyngeal swabs (NPS) from 11 cattle.

	Animal ID										
Plate number	**205**	**232**	**260**	**277**	**256**	**262**	**56–8**	**53–49**	**49–16**	**54–8**	**54–211**
1	8	0	4	14	13	6	8	17	0	10	9
2	11	0	7	15	5	0	19	19	4	11	13
3	7	0	10	15	11	0	12	18	3	8	12
4	16	0	9	14	15	0	6	20	2	2	13
5	6	1	11	12	15	1	11	20	1	5	13
Total *M. haemolytica* Isolates	48	1	41	70	59	7	56	94	10	36	60
Number of AMR phenotypes	1	1	3	2	1	1	2	2	1	1	1
M. haemolytica genotype	1	1	2	2	1	2	2	2	2	2	2

Two NPS (one from each nostril) were together streaked onto the first quadrant of 5 consecutive blood agar plates (plate numbers 1 through 5), then the remaining three quadrants of each plate were streaked with a new sterile loop. Each individual colony (up to 20 for each plate) was subcultured once for determination of minimum inhibitory concentrations for 11 antimicrobials to determine AMR phenotype, and for MALDI-TOF MS to determine genotype.

peaks was also included to verify proper genotype classification (10). *Mannheimia haemolytica* genotypes 1 and 2 that are identified by this MALDI quick classifier model were previously described by Clawson et al. (11), with genotype 2 isolates primarily originating from the lungs of cattle with clinical or pathological signs of respiratory disease, and typically harboring integrative conjugative elements (ICE) conferring multi-drug antimicrobial resistance, and genotype 1 isolates originating from the upper respiratory tract of cattle with no signs of disease, and typically not including ICE. For strains where there is genomic information available, genotype 1 strains are likely serotype 2 based on molecular analysis and genotype 2 strains are either serotype 1 or serotype 6 based on the same analysis, suggesting a strong relationship between serotype and genotype (12).

RESULTS

Animals and Bacterial Culture Results

Nasopharyngeal swabs were collected from cattle in four different groups (A–D) between March - May 2018 or in May 2019. Seven cattle were sampled in group A, six cattle were sampled in group B, five cattle were sampled in group C, and 10 cattle were sampled in group D. The seven cattle in group A were each sampled on two different occasions 15 days apart, but *M. haemolytica* was isolated from each animal only once, or not at all (four cattle positive when sampled the first time, two cattle positive when sampled the second time, and one animal negative at both sampling times). Cattle in groups B–D were only sampled once. Nasopharyngeal swabs from 11 of the 28 cattle yielded at least one *M. haemolytica* isolate. Details regarding the 11 cattle from which *M. haemolytica* were isolated are presented in **Table 1**. Two cattle (205 and 260) had not been treated with antimicrobials at the time of sampling, but they were treated for BRD based on clinical signs 2 days after they were sampled; none of the other cattle in that group (group A) were ever treated for BRD in the approximately 90-day period during which they were monitored. For each animal the median number (range) of *M. haemolytica* isolates obtained from the first plate streaked was 8 (0 −17), and the median number (range) of isolates from all 5 plates streaked was 48 (1–94) (**Table 2**). *M. haemolytica* was not identified on the first plate streaked for two of the cattle, and for one of these two cattle (232), only one *M. haemolytica* isolate was identified, on plate 5.

Phenotypes of Isolates

The AMR phenotype, or antibiogram, of each *M. haemolytica* isolate from each NPS was defined by the antimicrobial susceptibility to each antimicrobial tested in the broth microdilution assay. Isolates were defined as resistant or not resistant, with isolates having MIC in the intermediate range included with isolates in the sensitive range. The AMR phenotypes identified in the *M. haemolytica* isolates from each of the 11 animals described in **Table 2** are presented in **Figure 1**, and the MIC data for all antimicrobials for each isolate are presented in **Supplementary Material 1**. Nasopharyngeal swabs from 7 cattle yielded *M. haemolytica* with only one phenotype, NPS from 3 cattle yielded M. haemolytica with 2 phenotypes, and an NPS from one animal yielded *M. haemolytica* with 3 phenotypes. Differences in MIC among isolates from an individual animal that led to changes in phenotype were found for penicillin, tetracycline, or tilmicosin (**Supplementary Material 1**). However, when more than one phenotype was identified among the *M. haemolytica* isolates from the NPS from an individual animal, the difference between the phenotypes was always due to a difference of only one dilution near the breakpoint, which led to some isolates from an animal being defined as sensitive while others were identified as resistant. Since a difference of one dilution can be interpreted to be within the error of the broth microdilution assay, in this study all *M. haemolytica* isolates from the same NPS had essentially the same AMR phenotype.

Genotypes of Isolates

All isolates confirmed to be *M. haemolytica* by both Sensititre and by MALDI-TOF MS were assigned to genotype 1 or 2 based on the MALDI-TOF MS profile as described (10). All isolates obtained from the NPS from an individual animal were the same genotype. The isolates from NPS from 3 cattle were genotype

1, and the isolates from NPS from 7 cattle were genotype 2 (**Tables 1**, **2** and **Supplementary Material 1**).

DISCUSSION

Planning research to evaluate AMR in BRD leads to a recurring question: "How many *M. haemolytica* colonies do we need to select from a primary culture plate to have confidence that we have identified all the relevant isolates?". This research was undertaken to address this question. In work evaluating gamithromycin susceptibility of *M. haemolytica* isolates from bovine NPS or bronchoalveolar lavage fluid samples, Capik et al. (13) found that, when up to 12 *M. haemolytica* colonies were selected from primary plates, a mixture of sensitive and resistant isolates was sometimes found. However, that report did not provide exact numbers of sensitive and resistant isolates identified in individual samples, and it did not provide information for antimicrobials other than gamithromycin. To our knowledge no other research has described the number of different AMR phenotypes that can be identified in *M. haemolytica* isolated from the same bovine respiratory sample.

The number of colonies that need to be selected from a primary culture plate to accurately represent the diversity of isolates on the plate has been estimated for other bacterial pathogens. Singer et al. (5) developed a model to predict the number of isolates that need to be tested to determine with a high level of confidence the diversity of *Escherichia coli* (*E. coli*) isolates from cases of avian cellulitis. In this work the *E. coli* phenotype was defined by DNA pulsed field gel electrophoresis, and the model developed indicated that if 3 randomly selected colonies were phenotypically identical, the probability was 98.8% that only one phenotype was present on the plate. In other research, Döpfer et al. (6) developed a model to predict the number of isolates that need to be selected to identify all phenotypes of *E. coli, Listeria monocytogenes, Klebsiella pneumoniae,* or *Streptococcus uberis* on a culture plate, with phenotype defined by ribotyping, pulsed-field gel electrophoresis, or PCR-based strain-typing methods. The model of Döpfer et al. indicated that, for the bacteria evaluated, between 2 and 20 isolates needed to be selected and characterized to identify all phenotypes in the sample with 95% certainty. While the models of Singer et al. or Döpfer et al. should be applicable to *M. haemolytica*, they are based on Bayesian inferences that require an estimate of the number of different phenotypes ("prior information" or "prior probability") expected. The work presented here was undertaken to obtain this prior information, so that such models could be used to estimate the number of *M. haemolytica* colonies that need to be selected from a plate to provide a high level of confidence regarding the number of AMR phenotypes on the plate. However, the results indicated a surprising uniformity of phenotype, with essentially no diversity, and therefore the data did not support the use of a model to predict diversity. Since the phenotypes and genotypes of isolates from a sample were quite uniform, it appears that selecting one isolate may indeed adequately represent the characteristics of *M. haemolytica* isolates from bovine NPS. Put another way, the data suggest that a very large number of isolates would need to be tested to identify rare diverse isolates, which may not be feasible in terms of logistics or cost.

In addition to uniformity of AMR phenotype, the samples evaluated here revealed uniformity of *M. haemolytica* genotype, which was identified by the MALDI-TOF MS profile (10). This finding is similar to the results reported by Capik et al. (13), who showed that, when up to 12 *M. haemolytica* isolates were selected from culture plates from individual bovine NPS or bronchoalveolar lavage fluid samples, DNA sequencing and construction of phylogenetic trees revealed more than one genotype in only one of 12 samples described. In other work by the same group, characterization of multiple *M. haemolytica* isolates from the same bovine NPS culture showed little diversity as defined by DNA pulsed-field gel electrophoresis (14). Similarly, characterization of plasmid types from up to 8 *M. haemolytica* isolates from nasal swabs from feedlot cattle with or without BRD revealed that fewer than 10% of samples yielded more than one plasmid type (15), and evaluation of at least three *M. haemolytica* isolates from nasopharyngeal swabs from feedlot cattle demonstrated that isolates were in most cases identical based on pulse field gel electrophoresis (16). It should be noted that none of the genotyping methods used to characterize diversity of *M. haemolytica* isolates obtained from a single bovine respiratory sample, including our use of the MALDI quick classifier model to identify genotypes 1 and 2 described by Clawson et al. (11), provide the same resolution as whole genome sequencing. The genotyping approach used in this report is more similar to serotyping, where strains are classified broadly based on >26,000 nucleotide polymorphisms and have associations with capsular genes. Therefore, some differences among these apparently uniform isolates may have been present that would have been identified by whole genome sequencing or typing methods with higher resolution.

It has been reported (11) that genotype 2 *M. haemolytica* are most often isolated from cattle with clinical signs of BRD, while genotype 1 isolates are most often isolated from cattle that are clinically healthy. The results of the present study were generally consistent with this pattern, in that NPS from five of five cattle sampled at the time of BRD treatment yielded a genotype 2 *M. haemolytica*. Of the six cattle sampled when not showing signs of BRD, three cattle yielded a genotype 1 *M. haemolytica*, while genotype 2 *M. haemolytica* was isolated from the other three. However, one of the three "non-BRD" cattle with a genotype 2 *M. haemolytica* (animal 260) was treated for BRD 2 days after it was sampled.

In this study, NPS were streaked onto the first quadrant of five consecutive plates, in order to increase the likelihood of finding diverse *M. haemolytica* isolates that might have been overgrown by other bacteria on the first plate. This technique is not a standard practice in diagnostic laboratories, but given the fact that *M. haemolytica* was not identified on plate 1, but was identified on subsequent plates for two of the 11 cattle from which *M. haemolytica* was identified, the approach may be warranted in research. The lack of diversity across plates for each sample suggests that, once *M. haemolytica* is identified on one plate, the phenotype and genotype are likely to be similar to those identified on another plate from the same sample.

Limitations of this study include the relatively small number of cattle and cattle groups sampled, and the fact that sampled cattle came from a relatively limited geographic region. Given the lack of diversity found in this study, and the cost of characterizing large numbers of isolates from a single sample, it may be difficult to justify the cost to repeat this research with a larger number of cattle or groups. However, it is important to note that the results reported here may not be representative for other types of respiratory samples (e.g. nasal swabs or bronchoalveolar lavage samples), or for samples from other types of cattle, such as dairy calves, or for other BRD agents, such as *Pasteurella multocida* or *Histophilus somni*. Confirmation of the diversity of respiratory isolates as related to these other variables will require additional research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Mississippi State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AW, RW, and HC: Experimental design. HC, JL, and WE: Data collection. RW, MS, AT, and RS: Analysis of data. BK and WE: Animal maintenance and supervision of sample collection. All authors contributed to review and editing of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.883389/full#supplementary-material

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The prevalence of bovine mastitis-associated *Staphylococcus aureus* in China and its antimicrobial resistance rate: A meta-analysis

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In this study, to optimize the *Staphylococcus aureus* control program, a meta-analysis was conducted to investigate the epidemiology and antimicrobial resistance (AMR) profile of *S. aureus*-associated bovine mastitis in China from 2000 to 2020. A total of 33 publications from PubMed, Google Scholar, and China National Knowledge Infrastructure (CNKI) database were included in our research, among which nine publications included the AMR test. The pooled prevalence of *S. aureus* was 36.23%, and subgroup analysis revealed that the prevalence dropped from 2000–2010 to 2011–2020, which shows that China is on the right track. The pooled AMR rate indicate isolates were most resistant to β-lactams (50.68%), followed by quinolones (36.23%), macrolides (34.08%), sulfonamides (32.25%), tetracyclines (27.83%), aminoglycosides (26.44%), lincosamides (23.39%), and amphenicol (10.33%). Both the pooled prevalence and AMR of *S. aureus* in China are higher than those in Western countries, such as Germany, Belgium, Ireland, and the United States—countries with a long animal husbandry history and good management. Thus, there is still room to improve the treatment of *S. aureus*-associated bovine mastitis in China.

KEYWORDS

bovine mastitis, *Staphylococcus aureus*, prevalence, antimicrobial resistance, meta-analysis

Introduction

Bovine mastitis, as one of the most devastating diseases in dairy herds worldwide (1–3), is caused by several pathogenic bacteria, including *Staphylococcus aureus*. *Staphylococcus aureus* is one of the most prevalent pathogens worldwide and causes subclinical infections, resulting in an increased somatic cell count and intramammary

infections in dairy cows (4). *S. aureus* mastitis impacts dairy farms economically because of decreased productivity, premature culling, and prolonged costly antibiotic treatments (5–7).

The resistance of *S. aureus* to antimicrobials is a growing concern, along with its wide use against the disease, although the overall resistance rates vary widely by region (8). The standard treatment regimen against bovine mastitis with antibiotics is still under debate (9). China has greatly engaged in the global action plan on antimicrobial resistance (AMR) control (10). The National Action Plan to Combat Animal Origin Antimicrobial Resistance (2017–2020) (Beijing: China Ministry of Agriculture and Rural Affairs, 2017) is one of the national protocols to standardize veterinary medications in combination with strict biosecurity measures and prudent use of antimicrobials to alleviate the pressure of resistant pathogen transmission. Significant progress has been made against the AMR by prohibiting certain antibiotics (Announcement No. 194 of the Ministry of Agriculture and Rural Affairs of the People's Republic of China), for instance, officially prohibition of the use of three veterinary drugs, namely, olaquindox ["Chinese Veterinary Pharmacopeia" (2005 Edition)], clenbuterol (Notice of the General Office of the Ministry of Agriculture and Rural Affairs on Launching the Special Rectification Action for "Clenbuterol"), and salbutamol [State Pharmacopeia Commission. 2010 Pharmacopeia of the People's Republic of China (Part 2)] in food animals to ensure the quality and safety of animal products and maintain public health and ecological safety.

The prevalence and AMR rate of *S. aureus*-related bovine mastitis in different regions of China during 2000–2020 were estimated using meta-analysis (11), an innovative tool, by analyzing the findings of published studies. Pooled prevalence and AMR rate, as well as subgroup analysis, from different aspects were conducted.

The purpose of this study was to understand the epidemiology and AMR profiles of *Streptococcus* spp. using meta-analysis to optimize *Streptococcus* spp. control programs.

Materials and methods

Literature search

Literature retrieval steps and results are illustrated in Figure 1. A comprehensive and systematic literature search was conducted to identify studies on *S. aureus*-related bovine mastitis, utilizing PubMed (www.pubmed.gov), Google Scholar (https://scholar.google.com), and China National Knowledge Infrastructure (CNKI) database (https://www.cnki.net/). "Bovine mastitis AND bacteria" were used as key words fothe search of publications in English and Chinese between 2000 and 2020.

Selection of published studies

The PRISMA reporting standard was adopted in this study, as previously reported (12–14). Articles were excluded if (a) they were duplicate records; (b) they went off-topic and had small sample size (<3); (c) the study did not involve bacterial identification; (d) the study samples contained non-mastitis diseases; (e) the study involved ambiguous sample size or bacterial isolate quantity, and (f) the study was conducted out of the defined period (before 2000 and after 2020). Microsoft Excel was used to manage the references (Table 1).

Data extraction and statistical analysis

Designed forms were used to extract data from the selected publications, and the data included author, year of publication, province, sample size, number of *S. aureus* isolates, degree of mastitis (as per the Laboratory Handbook on Bovine Mastitis, National Mastitis Council), identification method, number of resistant isolates, and laboratory procedure. The methodological quality of each study was independently reviewed by two reviewers based on pre-specified study quality indicators adapted from the Downs and Black checklist.

The number of *S. aureus*, antimicrobial-resistant isolates, and mastitis milk samples of the extracted data were calculated for their proportion in articles. Resistance was considered a dichotomous outcome. The prevalence and AMR rate were separately meta-analyzed by using the "meta" and "metafor" packages in R (version 4.0.5).

The prevalence of *S. aureus* was pooled using the random effects model. Subgroup meta-analyses were conducted on isolation time and region, and mastitis grade to illustrate the heterogeneity between the studies.

The AMR profile was analyzed by groups: β-lactams, quinolones, aminoglycosides, tetracyclines, lincosamides, sulfonamides, macrolides, and amphenicol. The publication bias test was performed by using the Egger test, and a funnel plot was created.

Results

Inclusion of publications

A total of 34, 86, and 136 articles were obtained from PubMed, Google Scholar, and CNKI, respectively, among which the following were excluded: 24 publications were duplicates,

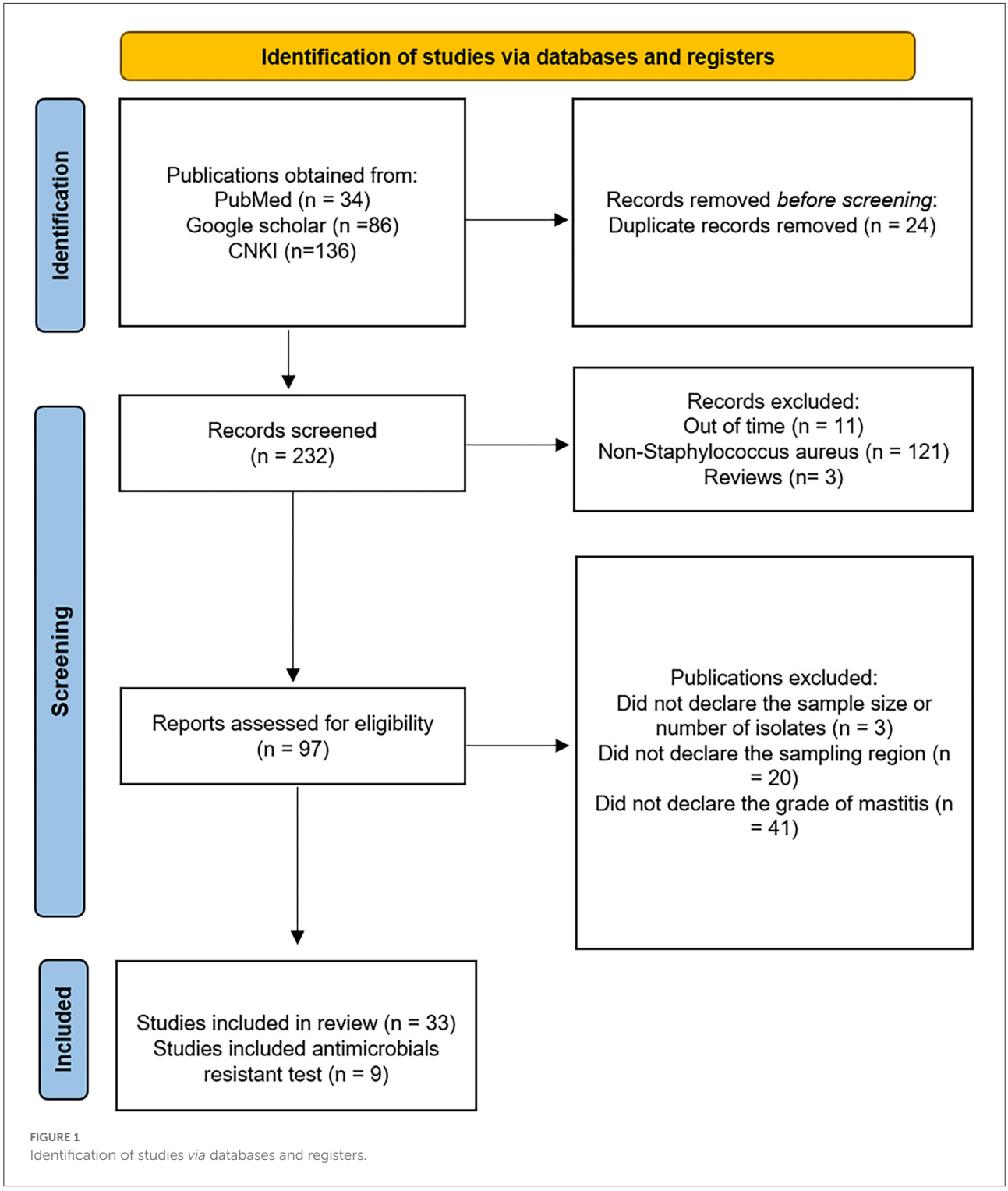

FIGURE 1
Identification of studies *via* databases and registers.

11 were published out of the defined period, 121 did not involving *S. aureus*, three were reviews, three did not provide information of sample size or the number of bacterial isolates, 20 did not provide data on the sampling region, and 41 did not provide grade of mastitis. As a result, 33 publications including 4,215 samples and 1,305 isolates were selected for subsequent analysis, of which nine were included for the AMR test (Figure 1, Table 1).

TABLE 1 Information of studies included in our study.

Author	Year	Sample	Identification assay	Isolates	Grade[a]	Region[b]	AMR method[c]
Meng Dan	2019	186	16S	98	C	N	
Weijie Jin	2020	544	16S	168	C	S	K–B
Lili Zhang	2016	200	Other	58	C	S	K–B
Qiang Ren	2019	84	16S	65	S	N	K–B
Feng Li Yang	2014	67	Other	12	C	S	
Chenchen Shen	2017	28	Other	18	C	S	K–B
Mingxu Zhou	2019	50	16S	5	S	S	K–B
Huiyun Zhao	2020	110	16S	15	C	N	K–B
Weize Gan	2020	812	16S	216	S	N	K–B
Haiyan Wu	2019	50	Other	18	C	N	K–B
Lijun Wu	2019	165	16S	43	S	S	
Wei Liu	2006	60	Other	43	C	N	
Lei Liu	2009	92	Other	58	S	N	
Yu Li	2011	16	Other	12	C	N	
Jin Li	2014	58	Other	53	C	N	
Lin Wang	2015	100	Other	15	C	N	
Hongwei He	2015	14	Other	12	C	N	
Xiujuan Ye	2004	44	Other	30	C	S	
Jianbiao Lu	2006	63	Other	23	C	N	
Ying Liu	2008	90	Other	23	C	N	
Guiying Wang	2008	115	Other	12	C	N	
Yongxin Yang	2009	86	Other	42	S	S	
Lulu Qin	2009	30	Other	4	C	S	
Guixian Zhang	2010	34	Other	6	C	N	
Fu Cong	2007	304	Other	91	S	N	
Long Ma	2009	44	Other	29	C	N	
Zhuming Zhang	2009	9	Other	5	C	N	
Xiaodong Kang[a]	2014	94	Other	7	C	N	
Xiaodong Kang[b]	2014	164	Other	11	C	N	
Jie Lin	2015	15	16S	10	C	N	
Xinpu Li	2015	302	16S	18	C	N	
Qiuyun Zhao	2016	48	Other	10	C	N	K–B
Liming Chen	2004	23	Other	12	C	S	
Yan Liu	2012	114	Other	63	C	S	
Total		4,215	–	1,305	–	–	–

[a]C, clinical bovine mastitis; S, subclinical bovine mastitis. [b]S, South China; N, North China. [c]K–B, disk diffusion test; –, publication did not include the AMR test.

Prevalence of *S. aureus*

The pooled prevalence of *S. aureus* is 36.23% [95% confidence interval (CI): 29.31–43.76%]. An evident heterogeneity was observed ($I^2 = 94\%$, $t^2 = 0.7583$, $P < 0.01$). Therefore, subgroup analysis was conducted to explore the sources of heterogeneity (Figure 2).

Subgroup analysis

The research articles were divided into subgroups based on research period (2000–2010 vs. 2011–2020), sampling sites (North vs. South China), and mastitis grade (clinical vs. subclinical mastitis). The pooled subgroup prevalence of *S. aureus* was 36.56% and 35.75% in North and South China,

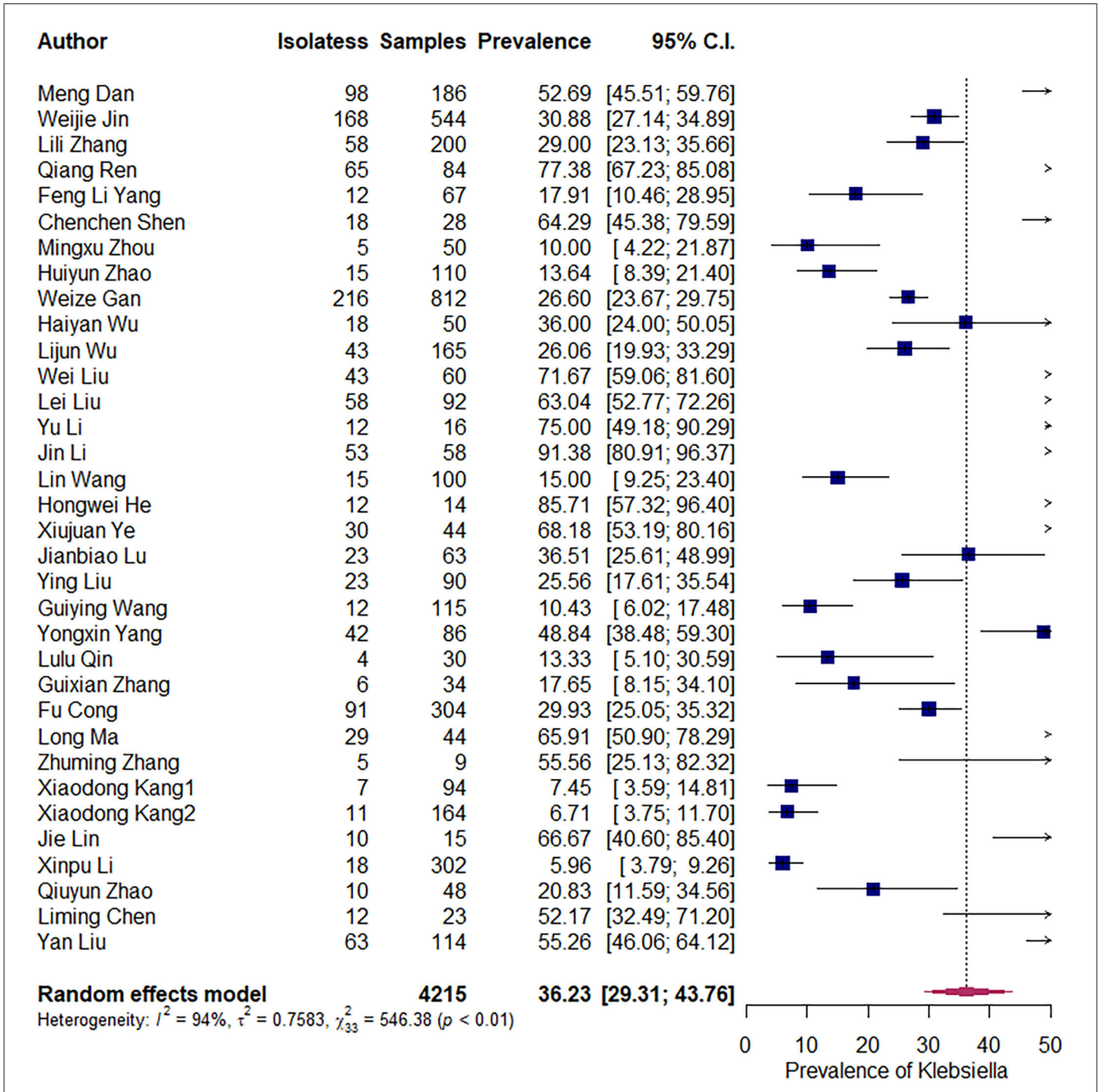

FIGURE 2
Overall prevalence of *Staphylococcus aureus*.

respectively (Figure 3); 43.22 and 32.62% for the 2000–2010 period and the 2011–2020 period, respectively (Figure 4); and 35.62% and 38.79% in clinical and subclinical mastitis, respectively (Figure 5).

According to the aforementioned meta-analysis results, we speculate that the difference in prevalence between South China and North China and the difference between periods 2000–2010 and 2011–2020 may be related to the difference in climate between North and South China and the increased emphasis on *Streptococcus agalactiae*, which is related to factors such as the improvement of biological prevention and control.

Antimicrobial resistance rate of *S. aureus*

The pooled antimicrobial resistant rate revealed that *S. aureus* was most resistant to β-lactams, 50.68% (95% CI: 42.55–58.77%); followed by quinolones, 36.23% (95% CI: 28.45–44.79%); macrolides, 34.08% (95% CI: 26.89–42.08%);

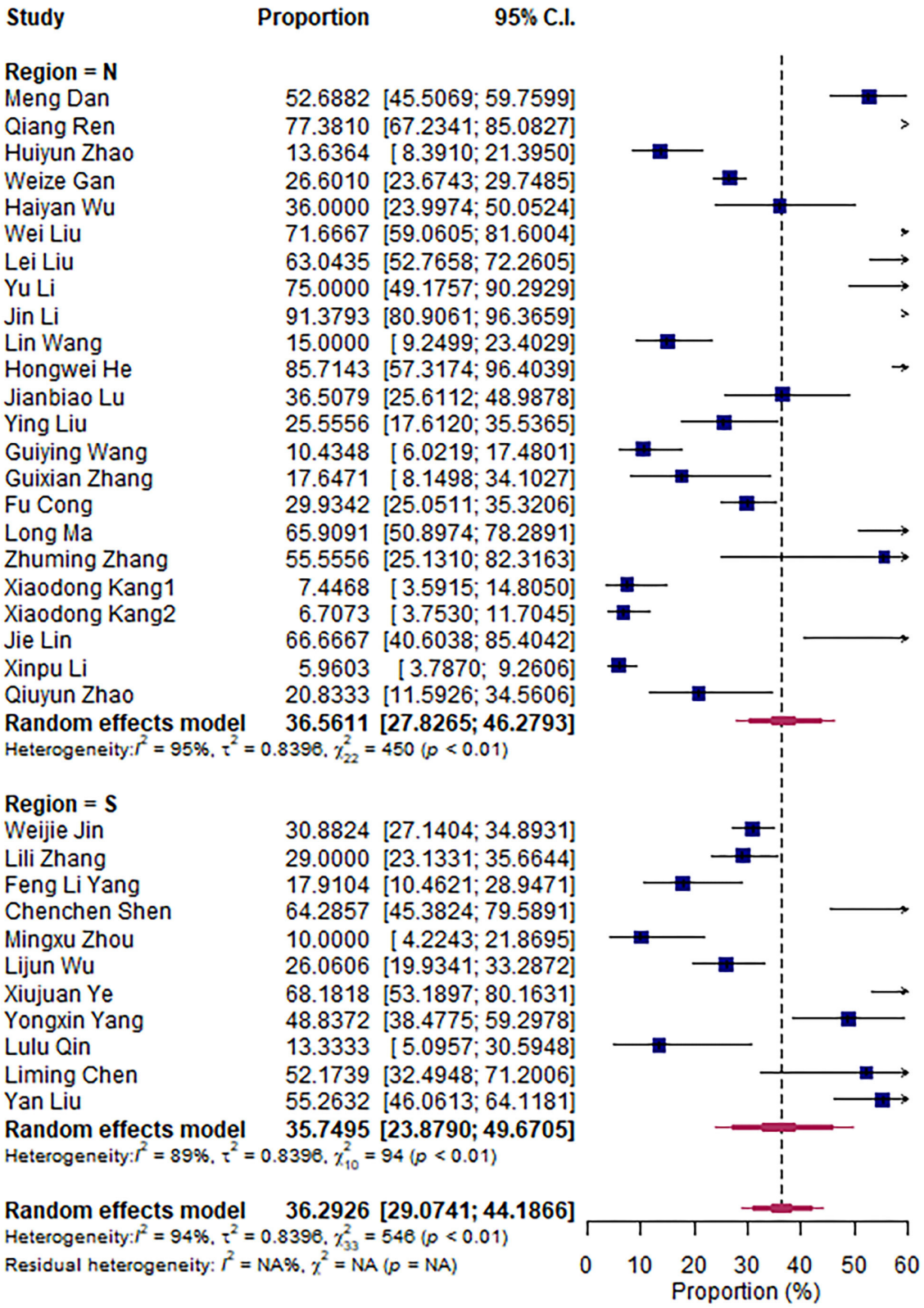

FIGURE 3
Prevalence subgroup by region.

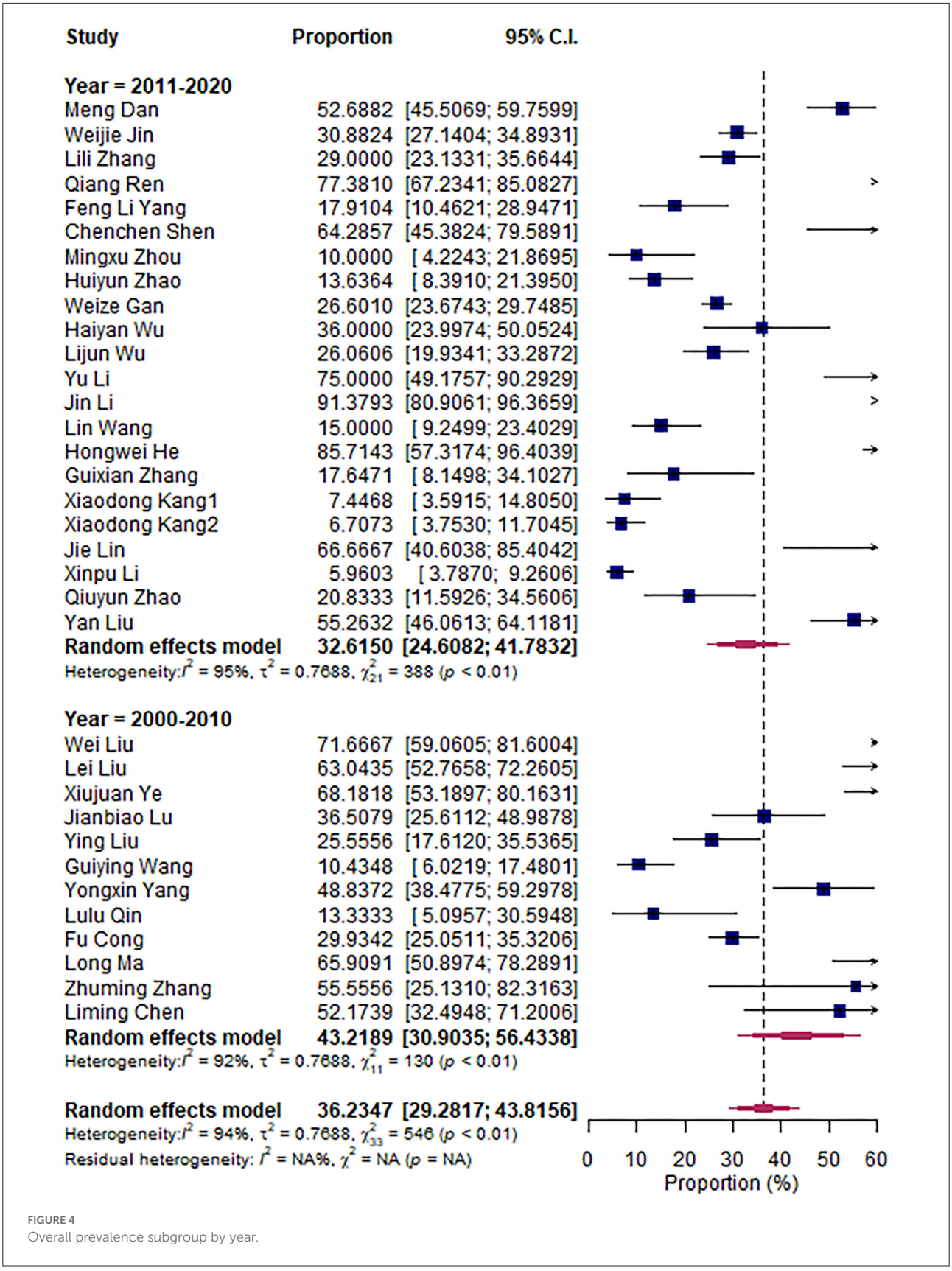

FIGURE 4
Overall prevalence subgroup by year.

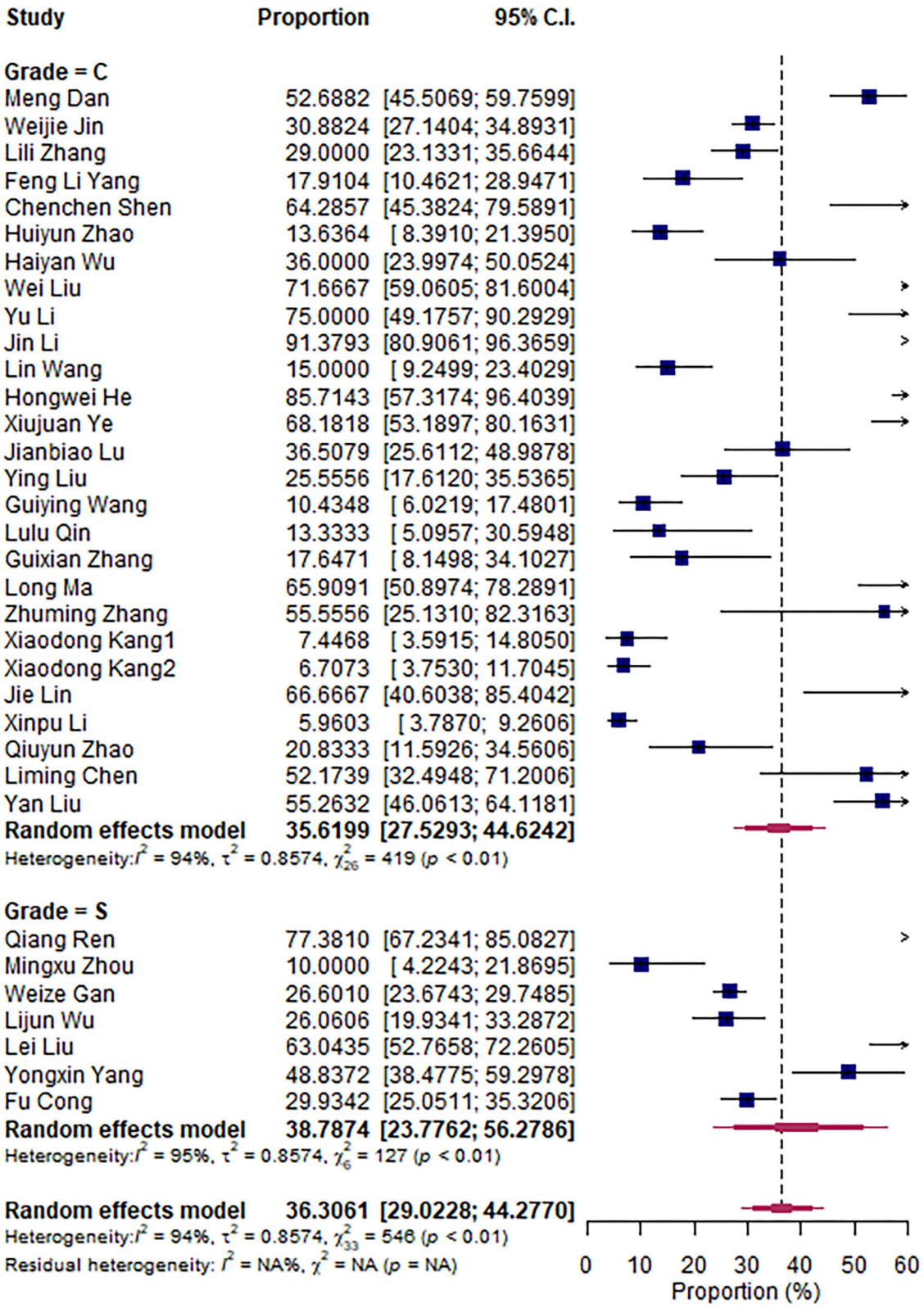

FIGURE 5
Overall prevalence subgroup by grade.

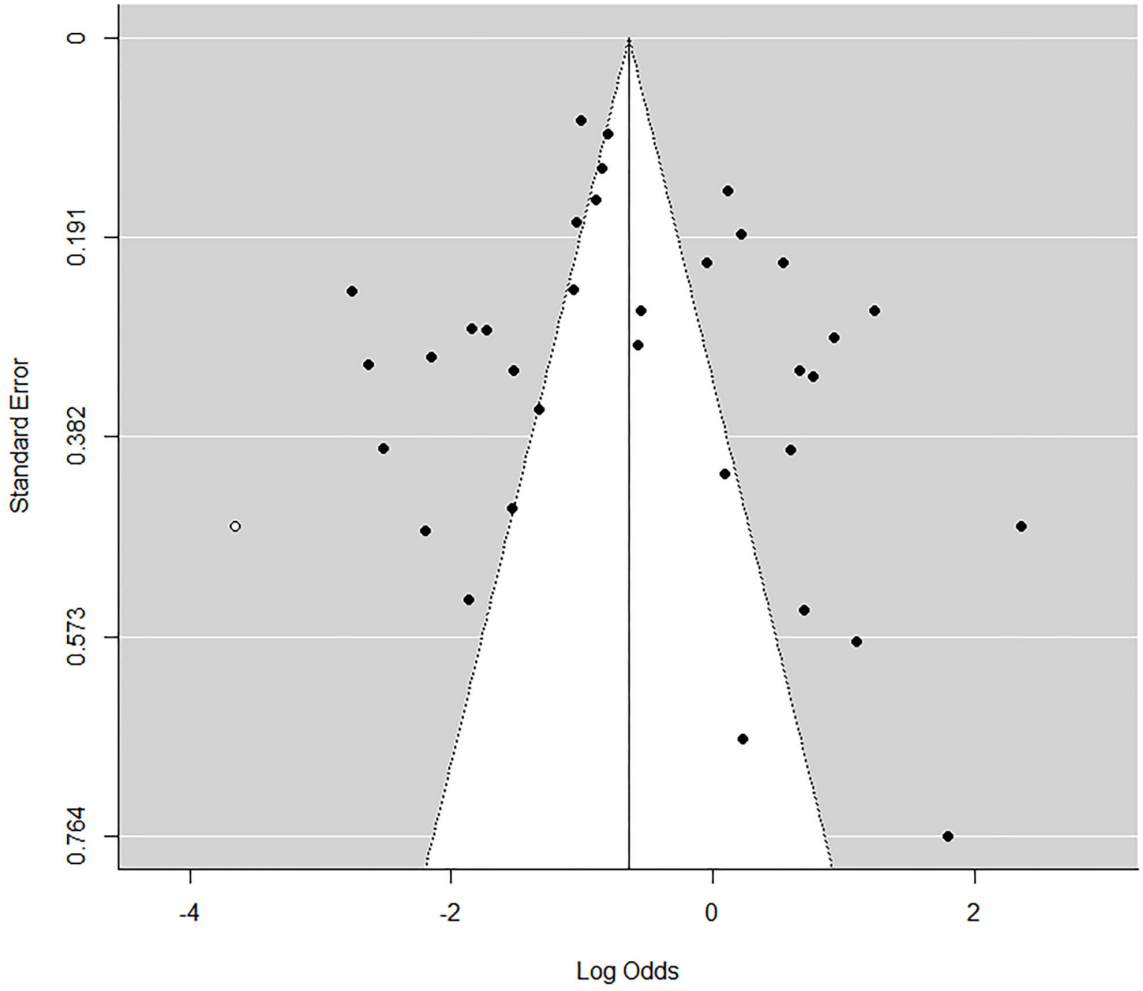

FIGURE 6
Antimicrobial resistance rate of *Staphylococcus aureus*.

sulfonamides, 32.25% (95% CI: 20.81–46.30%); tetracyclines, 27.83% (95% CI: 21.29–35.46%); aminoglycosides, 26.44% (95% CI: 19.33–35.02%); lincosamides, 23.39% (95% CI: 16.70–31.74%); and amphenicol, 10.33% (95% CI: 6.07–17.18%) (Figure 6).

Publication bias of the prevalence and AMR rate of *S. aureus*

As shown by the funnel plot (Figures 7, 8), the studies exhibited an even distribution around the mean effect size, which suggested the publication bias is negligible.

Discussion

Bovine mastitis is a disease of dairy cows worldwide (2, 15). *S. aureus* is one of the main pathogens causing the disease (16, 17) and is also the third largest foodborne pathogen in the world, posing a huge threat to animal husbandry and human public health (4), causing economic losses up to €300 per cow per year (18, 19), and thus fueling the increase of clinical, subclinical, and recurrent cow mastitis (6, 20). It is essential to understand the prevalence and AMR rate of bovine mastitis-related *S. aureus* to improve therapeutic interventions and prevention strategies.

The pooled prevalence of *S. aureus* in China (36.23%) is lower than that in the United States (46.6 ∼ 62.4%, 118 of 189 herds) (21), Hungary (70%) (22), Northern Greece (40%) (23), and northern Ethiopia (41.7%) (24), but is higher than that in Denmark (34%) (25), Germany (7.3 ∼ 11.5%) (26), Belgium (7.6%) (26), Iran (25%) (27), Japan (28.2%) (28), Nepal (15.2%) (29), and Korea (5.6%) (30). The difference in prevalence between China and the United States may be due to the fact that the scale of the United States is generally larger than that in China, and the farms covered in our study are only partial farms, and there may also be some high prevalence undetected. Apart from that, Patel et al. (21) suggested that caution should

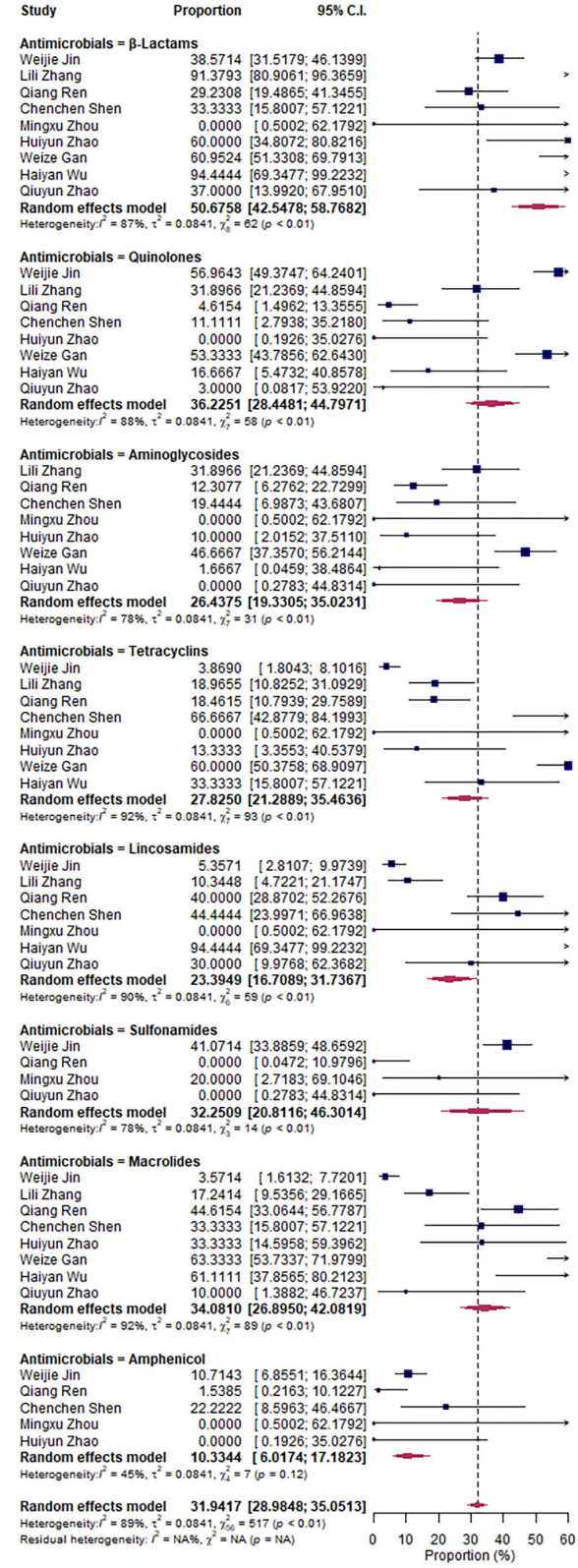

FIGURE 7
Funnel plot of prevalence bias of *Staphylococcus aureus*.

be exercised when generalizing the findings of smaller herds, so prevalence will still vary considerably between China and other developed countries. The fact that China is the third largest milk producer in the world may be the reason for the higher prevalence. It could be concluded that further measures need to be taken against bacterial resistance and to improve related managements in farms (2, 31, 32).

Song et al. (32) and Gao et al. (31) suggested that the higher prevalence in North China may due to colder winters and lack of heat, reluctance to keep up with the rapid development of farming technology and so forth. Another important reason may lie in the fact that the dairy industry is much more developed in North China, where the main dairy zone and the large-scale farms are located. However, in the study by Gao et al. (31), some samples were stored at 4°C, instead of a freezer; repeated freeze–thaw of the sample reduces the culture sensitivity of the bacteria. In addition, as mentioned in the study of Gao et al. we were unable to interpret the findings because of the lack of management details of the studied herds (31).

In our analysis, the prevalence of subclinical mastitis caused by *S. aureus* is higher than that of clinical mastitis, which is inconsistent with the fact that clinical mastitis is more common than subclinical mastitis (32) but is consistent with the fact that a higher incidence of subclinical mastitis is predominant (33). Meanwhile, the incidence of the clinical type of bacteriologic bovine mastitis was roughly 20 ∼ 22% in Canada (34), and that of subclinical mastitis was about 20.8 ∼ 23.3% in the United States (35). Moreover, considering the different research angles and the limitations of the sample size used in the analysis, the difference is not surprising (34, 35). The specific prevalence of clinical and subclinical mastitis in China requires further meticulous studies to draw more accurate conclusions.

The lower prevalence in the recent decade of 2011–2020 than the decade of 2000–2010 (32.62 vs. 43.22%) might imply the decline in the prevalence due to the rapid technology development against *S. aureus* and biosecurity measures undertaken by the farms. This may be a good sign that *S. aureus* could be more effectively controlled in future along with the development of more advanced technology and increased attention paid to the industry (36). Mammary gland health is further complicated by differences in farm management systems, farm sizes, cow cleanliness, and housing styles across countries and regions (37).

In our study, *S. aureus* is the most resistant bacterium to β-lactams (50.68%). It was shown in the study by Perovic that *S. aureus* may have an acquired gene that makes it resistant to methicillin and to all other β-lactam antibiotics (28, 38). In addition, penicillin belongs to the β-lactam class of drugs, the drug has been used for long-term and repeated administration in cattle, for example, for the treatment of diarrhea and other diseases, which may result in increased resistance to its use in the treatment of clinical mastitis (39); hence, β-lactams might

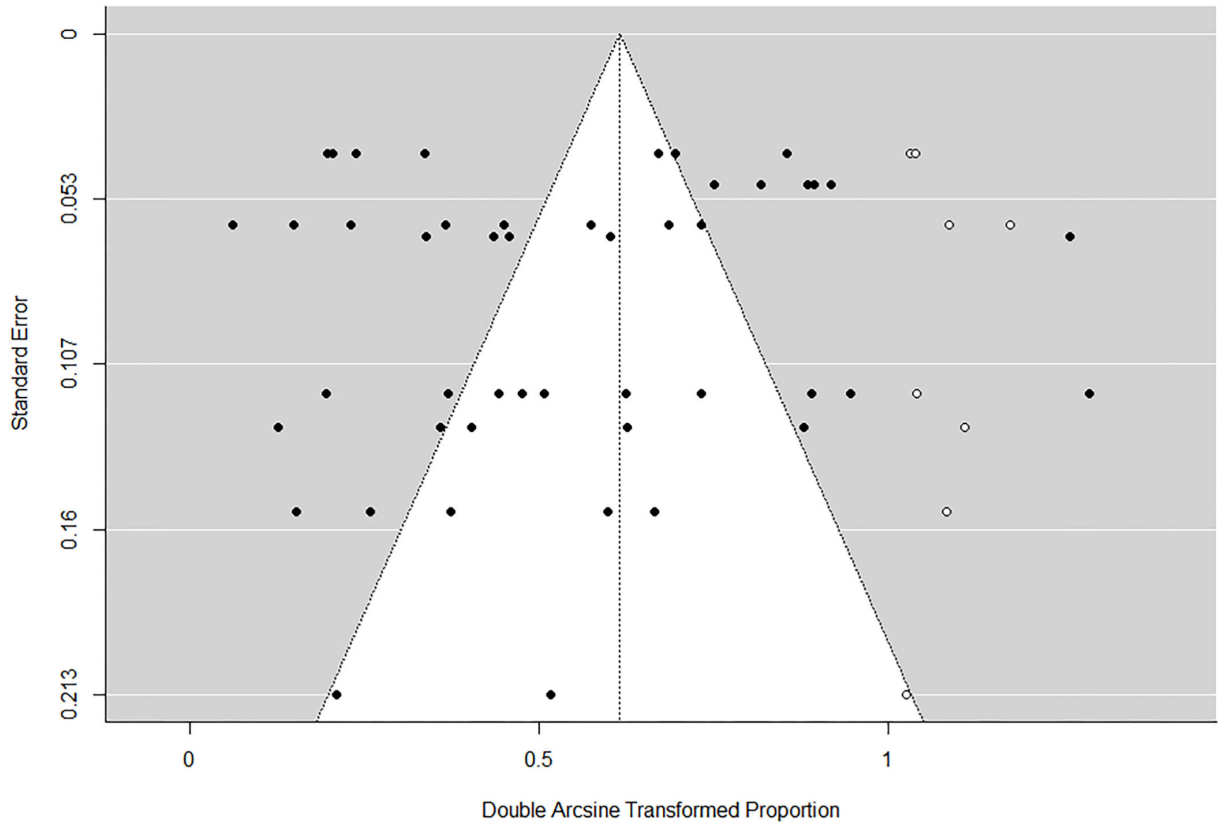

FIGURE 8
Publication bias of antimicrobial resistant rate of *Staphylococcus aureus*.

be the most resistant antibiotic against *S. aureus*. This result is supported by studies conducted in Iran (27) and Brazil (40), both showing that *S. aureus* is highly resistant to β-lactam antibiotics compared with other antibiotics. In India, resistance to oxacillin (a penicillin drug) can reach 20.5% (41). In Japan, the resistance to ampicillin can reach 76.1%∼89.7% (28). In Nepal, *S. aureus* isolates were totally (100%) resistant to ampicillin, 75.9% to cefazolin, and 48.3% to tetracycline (29).

China and other countries follow different practices regarding the use of antibiotics (42–44). However, rational evaluation, drug screening, and cautious and responsible use are meaningful to all countries to gradually reduce the use of antibiotics in veterinary practice in future.

Conclusion

The pooled prevalence of *S. aureus* was 36.23%, and subgroup analysis revealed that the prevalence was higher in North China in 2000–2010 and in subclinical bovine mastitis cases. Pooled AMR rates revealed *S. aureus* is highly resistant to β-lactams and quinolones; therefore, caution should be taken against treatments involving these two types of antibiotics for bovine mastitis.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

KW, JC, and KL contributed to conception and design of the study. KL organized the database. KW performed the statistical analysis and wrote the first draft of the manuscript. KL, JD, BY, HX, and JW wrote sections of the manuscript. LZ, CH, and XG performed the literatures research and review. WQ critically reviewed and revised the manuscript. All authors read and approved the final version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Detection of the dominant pathogens in diarrheal calves of Ningxia, China in 2021–2022

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Introduction: Calf diarrhea is a complex disease that has long been an unsolved problem in the cattle industry. Ningxia is at the forefront of China in the scale of cattle breeding, and calf diarrhea gravely restricts the development of Ningxia's cattle industry.

Methods: From July 2021 to May 2022, we collected diarrhea stool samples from calves aged 1–103 days from 23 farms in five cities in Ningxia, and performed PCR using specific primers for 15 major reported pathogens of calf diarrhea, including bacteria, viruses, and parasites. The effect of different seasons on the occurrence of diarrhea in calves was explored, the respective epidemic pathogens in different seasons were screened, and more detailed epidemiological investigations were carried out in Yinchuan and Wuzhong. In addition, we analyzed the relationship between different ages, river distributions and pathogen prevalence.

Results: Eventually, 10 pathogens were detected, of which 9 pathogens were pathogenic and 1 pathogen was non-pathogenic. The pathogens with the highest detection rate were *Cryptosporidium* (50.46%), Bovine rotavirus (BRV) (23.18%), *Escherichia coli* (*E. coli*) K99 (20.00%), and Bovine coronavirus (BCoV) (11.82%). The remaining pathogens such as Coccidia (6.90%), Bovine Astrovirus (BoAstV) (5.46%), Bovine Torovirus (BToV) (4.09%), and Bovine Kobuvirus (BKoV) (3.18%) primarily existed in the form of mixed infection.

Discussion: The analysis showed that different cities in Ningxia have different pathogens responsible for diarrhea, with *Cryptosporidium* and BRV being the most important pathogens responsible for diarrhea in calves in all cities. Control measures against those pathogens should be enforced to effectively prevent diarrhea in calves in China.

KEYWORDS

diarrhea, calf, epidemic investigation, Ningxia, pathogens

Introduction

Diarrhea is one of the most important diseases that damages the health of calves worldwide. It is considered to be one of the diseases causing the highest economic losses to the cattle industry, with losses of up to 10 million dollars due to calf diarrhea in Norway in 2006, followed by cases of varying degrees of calf diarrhea reported in the United States in 2007, South Korea in 2013, and Pakistan in 2014 (1, 2). The main causes of calf diarrhea are intricate and complex (3, 4). In addition to genetics, age, herd and farm environment, feeding practices, poor management and other complications, the most important factor is infection (5, 6). Many countries, including China, have experienced calf diarrhea outbreaks of differing degrees caused by pathogens, such as *Cryptosporidium*, BRV, BCoV,

E. coli K99 and other pathogens (7–10). According to the annual report of Japan in 2017, the economic losses caused by BRV in the previous years were estimated to be about 1 billion yen (11). In addition to causing diarrhea, *Cryptosporidium*, BCoV, and *E.coli* K99 also have different effects on increasing mortality, reducing immunity, and reducing milk production (12, 13).

In China, calf diarrhea outbreaks have been reported in many provinces and regions (14–17), but Ningxia has few reports in the article that has comprehensively and systematically investigated the epidemic situation and pathogen distribution characteristics of calf diarrhea. Ningxia has a natural and favorable breeding environment, coupled with the government policy support for the cattle breeding industry, making it one of the important cattle breeding areas in China. With the growing scale of the cattle industry in Ningxia, diarrhea in calves has become an increasingly serious problem, such as the absence of clinical symptoms in calves carrying the pathogen, the rapid spread of the pathogen, and the effect of different environments on the occurrence of diarrhea, which have not been reported or studied.

In order to investigate the prevalence of calf diarrhea in Ningxia and clarify the main pathogens that cause calf diarrhea prevalence in different cities, and study the effects of different seasons to diarrhea in calves, calf diarrhea fecal samples were collected from 23 large-scale cattle farms in five cities of Yinchuan, Wuzhong, Shizuishan, Zhongwei and Guyuan. Pathogens that have been reported to be associated with calf diarrhea were tested, including *E. coli* K99 (18), *Salmonella* (19), *Proteus mirabilis* (20), *Clostridium perfringens* (*C. perfringens*) (21), Bovine Viral Diarrhea Virus (BVDV) (22), BRV (23), BCoV (23), BToV (22), BoAstV (24), BKoV (24), Bovine Norovirus (BNoV) (24), Bovine Enterovirus (BEV) (25), *Cryptosporidium* (26), *Coccidia* (27, 28), and *Giardia* (29). The prevalence and distribution characteristics of these pathogens were analyzed to develop a reasonable and effective treatment plan for diarrhea in calves and to provide basic data for the prevention of diarrhea in calves.

Materials and methods

Sampling

From July 2021 to May 2022, 315 calf stool samples including 220 fresh calf stool samples with diarrhea and 95 fresh normal samples from 23 large-scale cattle farms in 5 cities of Ningxia were collected. Using sterile disposable gloves to collect normal calf rectal stool samples; 4 mL fetal calf serum(FBS)-free DMEM was taken to a sterile 15 mL tube, and the diarrhea stool samples were collected into the tube and stored at 4°C. The common symptoms of diarrheal calves were dehydration, loss of appetite, watery diarrhea, and mental depression. Figure 1A shows the geographical location of the Ningxia Hui Autonomous Region, and Figure 1B shows the geographical location of the cattle farm and the total number of samples collected in each area. Table 1 shows the specific sampling numbers in Ningxia.

DNA extraction

After the collected fresh stool was transported back to the laboratory at 4°C, 200 g of stool were dispensed into 2 mL sterile EP

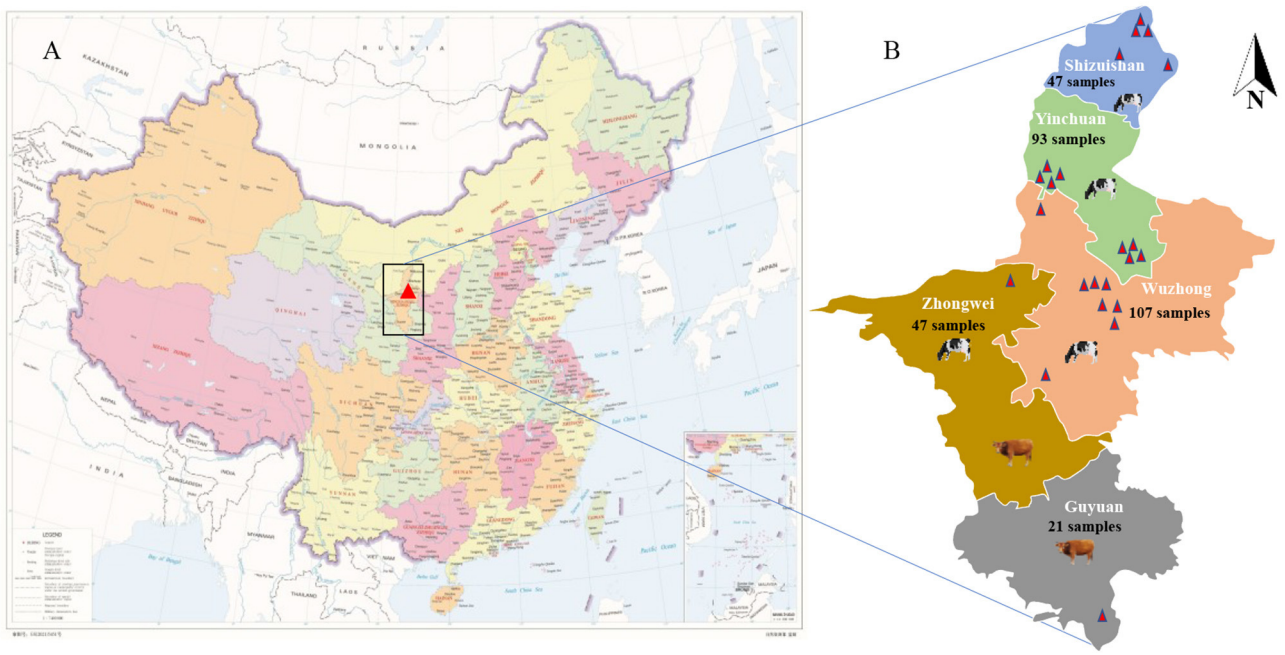

FIGURE 1
Cattle farm location and sampling information. **(A)** The geographical location of Ningxia Hui Autonomous Region (red marked as Ningxia Hui Autonomous Region, red star marked as Beijing, the capital of China). **(B)** The geographical location of the cattle farm and the total number of samples collected in each city (different colors represent different cities).

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TABLE 1 The total number of samples with diarrhea and non-diarrhea in five cities.

Location	Number of the calves of diarrhea	Number of non-diarrhea calves
Yinchuan	68	25
Wuzhong	70	37
Shizuishan	35	12
Zhongwei	38	9
Guyuan	9	12
Total	220	95

tubes on a sterile clean bench, and total DNA was extracted using stool DNA kit (OMEGA, Georgia, USA), and then PCR detection was performed to detect *E. coli* K99, *Salmonella*, *Proteus mirabilis*, *C. perfringens*, *Coccidia*, *Cryptosporidium*, *Giardia*.

RNA extraction and reverse transcription

The collected fresh diarrhea stool samples were diluted with 0.9% sterile normal saline. After repeated freezing and thawing at −80°C for three times, the samples were centrifuged at 4°C, 12,000 r/min for 5 min, and the supernatant was collected. Total RNA was extracted from stool using Trizol reagent *AG RNA ex Pro* (Accurate Biotechnology, Hunan, China). According to the manufacturer's operating rules, 2 μg total RNA was reverse transcribed into cDNA using *Evo M-MLV* RT Mix kit with gDNase. The cDNA was used to detect viruses that caused bovine diarrhea such as BVDV, BRV, BCoV, BToV, BoAstV, BKoV, BNoV, and BEV.

Identification and detection of pathogens by PCR

The primers used to detect the above pathogens are shown in Table 2. The extracted RNA was measured using NanoDrop One (Thermo Fisher Scientific, Waltham, MA, USA) and the RNA concentration was in the normal range. Each sample was taken 2 μg RNA for reverse transcription to obtain the same concentration of cDNA. Nested PCR was performed to detect *Cryptosporidium* and *Giardia* using 2 × Taq Master Mix (Vazyme Biotech, Nanjing, China), the specific PCR system was 2 × Taq Master mix 10 μL, upstream and downstream primers 1 μL, template 2 μL, supplemented with ddH_2O to 20 μL, the primer concentration was 10 μM. PCR amplification of other pathogens was carried out using 2 × M5 HiPer plus Taq HiFi PCR mix (Mei5 biotechnology, Beijing, China), the specific PCR system was 2 × M5 HiPer plus Taq HiFi PCR mix 10 μL, upstream and downstream primers 1 μL, template 2 μL, supplemented with ddH_2O to 20 μL, the primer concentration was 10 μM.

Detection *Coccidia*

Take 2 g stools sample of diarrheal calves (≥18 d), put it into a beaker, add 5 mL of water first, stir and mix well, add saturated saline to 60 mL, filter through a copper mesh after mixing, absorb the stool liquid, and inject it into McMaster Egg Slide Counting Chamber, after stewing for 5 min, count the number of EPG (Egg Per Gram) or OPG (Oocysts Per Gram) in the two graduated chambers under the microscope (27, 28).

The average A of the number of eggs in the two counting chambers multiplied by 200 is the number of eggs or oocysts per gram of stool. Compute the amount of EPG or OPG of oocysts per gram of stool according to the following formula:

$$EPG/OPG = [(n1 + n2)/(2\times0.15)]\times60\div2 = A\times 200$$

Statistical analysis

All PCR products were visualized on a 1.0% agarose gel. All positive samples were purified and sequenced by Tsingke Biotechnology (Beijing, China). The sequence results were aligned in GenBank.

The correlation between the pathogen detection rate and the distance between the cattle farm and the river was analyzed using GraphPad, version 9.0.0. Statistical analyses of pathogen detection rates in different seasons throughout Ningxia and in different seasons in Yinchuan and Wuzhong were performed using GraphPad, version 9.0.0. Chi-square tests were performed at a 5% level of significance in SPSS 20.

Results

Detection of different pathogens by PCR

Cryptosporidium

PCR detection using primers designed by Xiao et al. (6), 111 (50.46%) of 220 stool samples were positive, of which 53 (24.09%) were infected by *Cryptosporidium* alone, and the rest were mixed infection (26.36%). The two highest proportions of mixed infections were *Cryptosporidium* and *E. coli* K99 (5.91%), followed by *Cryptosporidium* and BRV (5.45%), and then *Cryptosporidium* and Giardia (4.09%).

Giardia

PCR detection using primers designed by Sulaiman (30), 30 (13.64%) of 220 stool samples were positive, of which 9 (4.09%) were infected by *Giardia* alone and the rest were mixed infection (9.55%). The two highest proportions of mixed infections were *Giardia* and *Cryptosporidium* (4.09%), followed by *Giardia* & *Cryptosporidium* & *E. coli* K99, *Giardia* & *Cryptosporidium* & BRV, *Giardia* & *Cryptosporidium* & BCoV, with a detection rate of 0.91%.

E. coli K99

PCR detection using the primers reported by Keykhaei (18), among the 220 stool samples, 44 (20.00%) were positive, of which

TABLE 2 Primers used for PCR.

Pathogens species	Primer	Sequence (5′-3′)	Product size (bp)	References
E. coli K99	*F5*	F: TATTATCTTAGGTGGTATGG	314	(18)
		R: GGTATCCTTTAGCAGCAGTATTTC		
BCoV	Nsp10 of ORF1a	F: CGAGTTGAACACCCAGAT	230	(23)
		R: GAGACGGGCATCTACACT		
BRV	VP6	F: CCACCAGGTATGAATTGGAC	231	
		R: GAGTAATCACTCAGATGGCG		
BNoV	RdRp	F: AGTTAYTTTTCCTTYTAYGGBGA	532	(20)
		R: AGTGTCTCTGTCAGTCATCTTCAT		
BKoV	3D	F: TGGAYTACAAGRATGTTTTGATGC	216	
		R: TGTTGTTRATGATGGTGTTGA		
BoAstV	ORF1a	F: GAYTGGACBCGHTWTGATGG	432	
		R: KYTTRACCCACATNCCAA		
BEV	5′-UTR	F: AGCAACACTGGATTGTGCG	416	(25)
		R: GGAGTAGTCCGACTCCGC		
BVDV	5′-UTR	F: GCTAGCCATGCCCTTAG	290	(22)
		R: CCATGTGCCATGTACAG		
BToV	M	F: TTCTTACTACACTTTTTGGA	603	
		R: ACTCAAACTTAACACTAG AC		
Cryptosporidium	18S rRNA F1	F: TTCTAGAGCTAATACATGCG	1325	(6)
	18S rRNA R1	R: CCCATTTCCTTCGAAACAGGA		
	18S rRNAF2	F: GGAAGGGTTGTATTTATTAGATAAAG	830	
	18S rRNA R2	R: AAGGAGTAAGGAACAACCTCCA		
Giardia	TPI AL3543	F: AAATIATGCCTGCTCGTCG	605	(30)
	TPI AL3546	R: CAAACCTTITCCGCAAACC		
	TPI AL3544	F: CCCTTCATCGGIGGTAACTT	530	
	TPI AL3545	R: GTGGCCACCACICCCGTGCC		

14 (6.36%) were infected by *E. coli* K99 alone, and the rest were mixed infection (13.64%). The two highest proportions of mixed infections are *E. coli* K99 and *Cryptosporidium* (7.73%), followed by *E. coli* K99 and BRV (4.09%), and the proportion of simultaneous infection of *E. coli* K99, *Cryptosporidium*, and BRV is 1.82%. In the stool samples in which *E. coli* K99 was detected, it was only coinfected with *Cryptosporidium* and BRV, and no other pathogens were detected.

Bovine rotavirus

Using the VP6 gene primers of BRV designed by Guo (23) for PCR detection, 51 (23.18%) of 220 stools were positive, of which 16 (7.28%) were infected by BRV alone, and the rest were mixed infection (15.91%). The two highest proportions of mixed infections were BRV and *Cryptosporidium* (7.73%), followed by BRV and *E. coli* K99 (4.09%), and then BRV and BCoV (2.73%).

Bovine coronavirus

Using the Nsp10 gene primers in ORF1a of BCoV designed by Guo (23), 26 (11.82%) of 220 stools were positive, of which 8 (3.64%) were infected alone and 18 (8.18%) were infected with mixed infection. The two highest proportions of mixed infections were BCoV and *Cryptosporidium* (4.55%), followed by BCoV and BRV (0.91%), and then BCoV and *E. coli* K99 (0.91%).

Bovine kobuvirus

Using the 3D gene primers of BKoV designed by Shi et al. (20) for PCR detection, 7 (3.18%) of 220 stools were positive, all of which were mixed infections. BKoV was predominantly coinfected with *Cryptosporidium* (1.82%) and BRV (1.36%).

Bovine astrovirus

PCR detection using the primers of the ORF1a gene of BoAstV reported by Shi (20) showed that 12 (5.46%) of

TABLE 3 The details of *coccidia* infections in calf diarrhea.

Cities	Number of detected calves	Number of infected calves	Infection rate (%)	OPG (pcs/g)	
				Range	Average
Wuzhong	23	2	8.70	4,100–8,600	6,350
Yinchuan	12	2	16.67	17,200–19,000	18,100
Shizuishan	6	0	0	0	0
Zhongwei	8	0	0	0	0
Guyuan	9	0	0	0	0
Total	58	4	6.90	4,100–19,000	12,225

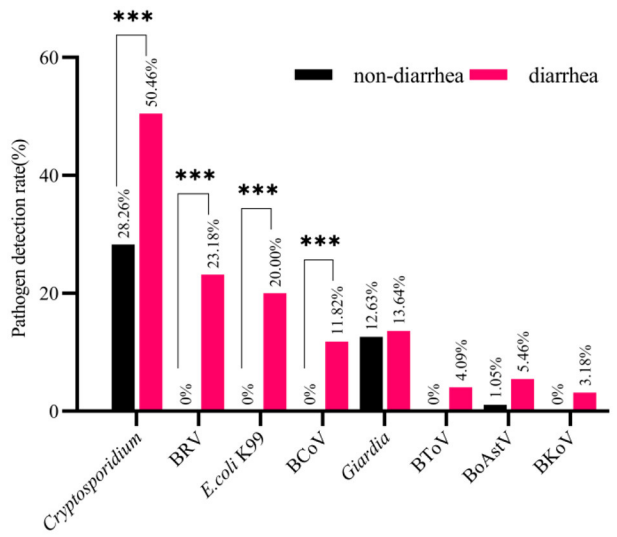


FIGURE 2
The Pathogens detection rate of diarrhea samples and the Pathogens detection rate of normal samples. The number of pathogen detection and non-detection in normal stool samples with the number of pathogen detection and non-detection in diarrhea stool samples were calculated, and then chi-square test was performed (mean ± standard deviation) ***$p < 0.01$.

220 stools were positive, of which 1 was infected alone (0.45%), and the rest were mixed infection (5.00%). The two highest proportions of mixed infections were BoAstV and *Cryptosporidium* (2.73%), followed by BoAstV and BRV (1.36%).

Bovine torovirus

The detection was executed using primers designed by Park (22) for the M gene of BToV to PCR. The results showed that 9 of 220 stools (4.09%) were positive, of which 1 was a single infection (0.45%), and the rest were mixed infections (3.64%), suggesting BToV is likely coinfected with two or more pathogens. In a sense, significant diarrhea symptoms only occur when BToV is coinfected with other pathogens.

TABLE 4 The details of calf diarrhea single infection.

Pathogens	Number	Percent (%)
Cryptosporidium	53	24.09
BRV	17	7.73
E. coli K99	14	6.36
Giardia	9	4.09
BCoV	8	3.64
BToV	1	0.46
BoAstV	1	0.46
Coccidia	1	0.46
BKoV	0	0
Total	104	47.27

Coccidia

Through the McMaster Egg Slide Counting Chamber, four cases (6.90%) of 58 calves (≥18 days) were detected positive for *coccidia* in this study, including two cases in Wuzhong and two cases in Yinchuan. The OPG levels of the two cases were 4,100 and 8,600 in WuZhong, and the calf ages were 89 and 84 d. The OPG content of the 2 cases was 17,200 and 19,000 in Yinchuan, and the age of the calf was 28 and 27 d. The specific results are shown in Table 3.

Together, a total of nine pathogens causing diarrhea including bacteria, viruses and parasites were detected in this study. The single infection rate of the detected pathogens is shown in Figure 2. The details of a single infection are shown in Tables 4, 5 for details of a mixed infection.

Detection rate of different types of pathogens

In this study, 220 stool samples of calves with diarrhea and 95 normal samples of calves were detected. Among bacterial pathogens, *E. coli* K99 and *C. perfringens* were detected, and *Proteus mirabilis* and *Salmonella* were not detected. The primers reported by Jiang (21) were used to identify *C. perfringens*. The *C. perfringens* detected in this study were all type A and had no pathogenicity.

TABLE 5 The details of calf diarrhea mixed infection.

Pathogens	Number	Percent (%)
E. coli K99 & *Cryptosporidium*	13	5.91
BRV & *Cryptosporidium*	12	5.45
E. coli K99 & BRV	9	4.09
Cryptosporidium & *Giardia*	9	4.09
BCoV & *Cryptosporidium*	7	3.18
BoAstV & *Cryptosporidium*	5	2.27
BRV & BCoV	3	1.36
BCoV & *Cryptosporidium* & *Giardia*	2	0.91
BRV & *Cryptosporidium* & *Giardia*	2	0.91
E. coli K99 & *Cryptosporidium* & *Giardia*	2	0.91
E. coli K99 & BCoV	1	0.45
BToV & BoAstV	1	0.45
BToV & *Giardia*	1	0.45
BoAstV & *Giardia*	1	0.45
BRV & *Giardia*	1	0.45
BToV & *Coccidia*	1	0.45
BKoV & *Cryptosporidium*	1	0.45
BRV & BCoV & BKoV	1	0.45
BRV & BToV & BKoV	1	0.45
BRV & BToV & BoAstV	1	0.45
BCoV & BKoV & *Coccidia*	1	0.45
E. coli K99 & BCoV & *Giardia*	1	0.45
E. coli K99 & BRV & *Cryptosporidium*	1	0.45
E. coli K99 & BoAstV & *Cryptosporidium*	1	0.45
BRV & BCoV& BoAstV& *Giardia*	1	0.45
BRV & BToV& BKoV& *Cryptosporidium*	1	0.45
E. coli K99 & BToV & BKoV& *Cryptosporidium*	1	0.45
BCoV & BKoV & *Coccidia* & *Cryptosporidium*	1	0.45
E. coli K99 & BRV & BToV & BoAstV & *Giardia*	1	0.45
Total	83	37.73

The detection rates of all pathogens from high to low are *Cryptosporidium* (50.46%), BRV (23.18%), *E. coli* K99 (20.00%), BCoV (11.82%), *Giardia* (13.64%), BoAstV (5.46%), BToV (4.09%), BKoV (3.18%). Comparison of detection details between diarrhea stool samples and normal stool samples by chi-square test, among them, the detection rates of *Cryptosporidium* ($p < 0.01$), BRV ($p < 0.01$), *E. coli* K99 ($p < 0.01$), and BCoV ($p < 0.01$) were significantly different between diarrhea stool samples and normal stool samples, while no significant differences were found for the other four pathogens including *Giardia* ($p = 0.859$), BoAstV ($p = 0.118$), BToV ($p = 0.062$), BKoV ($p = 0.107$).

Among the four diarrhea-related pathogens, BRV, *E. coli* K99, and BCoV were not detected in normal stool samples, but *Cryptosporidium* (28.26%) was detected in normal stool samples. The results showed that *Cryptosporidium* had a certain content in normal stool samples and diarrhea stool samples. No clinical diarrhea symptoms in normal stool samples were due to the low content of *Cryptosporidium* in calves. Among other pathogens, *Giardia* (12.63%) and BoAstV (1.05%) were also detected in normal samples and were present in the same situation as *Cryptosporidium*. In contrast, BToV and BKoV were not detected in normal samples, but the results were not significantly different from BToV (4.09%) and BKoV (3.18%) detection rates of diarrhea samples.

The detection of pathogen in different cities

A total of 68 stool samples with diarrhea were detected in Yinchuan: *E. coli* K99 was detected in 19 samples (27.94%); BRV in 14 samples (20.59%); BCoV in 6 samples (8.82%); BToV in 2 samples (2.94%); BoAstV in 1 sample (1.47%); BKoV in 2 samples (2.94%); *Coccidia* in 2 samples (2.94%); *Cryptosporidium* in 28 samples (41.18%); *Giardia* in 6 (8.82%) samples.

The Chi-square test showed that the main epidemic cause of diarrhea happened in Yinchuan was *E. coli* K99 ($p < 0.01$), followed by BRV ($p<0.05$). Although the detection rate of *Cryptosporidium* (41.18%) was the highest in diarrhea stool samples in Yinchuan, it was also the highest in normal stool samples, and the difference was not significant (32.00%). Other pathogens were detected in normal stool samples. The specific results are illustrated in Figure 3A.

Wuzhong detected *E. coli* K99 in 11 (15.71%) of 70 diarrhea stool samples; BRV in 12 (17.14%) samples; BCoV in 15 (21.43%) samples; BToV in 2 (2.86%) samples; BoAstV in 1 (1.43%) sample; BKoV in 4 (5.71%) samples; *Coccidia* in 2 (2.68%) samples; *Cryptosporidium* in 40 (57.14%) samples; *Giardia* in 14 (20.00%) samples.

The Chi-square test showed that the main epidemic pathogen causing diarrhea in Wuzhong calves was BCoV ($p < 0.01$), followed by BRV ($p < 0.01$), *E. coli* K99 ($p < 0.05$), *Cryptosporidium* ($p < 0.05$). Only *Cryptosporidium* was detected in both diarrhea and normal stool samples, and the other three pathogens were not detected in normal stool samples. Although the detection rate of *Giardia* in Wuzhong diarrhea stool samples was higher (20.00%), and it (13.51%) was second only to *Cryptosporidium* (35.14%) in normal stool samples, and the detection rate of *Giardia* in normal stool samples and diarrhea stool samples showed no difference. BToV and BKoV were not detected in normal samples, the detection rates in diarrhea stool samples were low (2.86%, 5.71%), and the difference was not significant. The detection rate of BoAstV in normal stool samples (2.70%) was even higher than that in diarrhea stool samples (1.43%). The results are detailed in Figure 3B.

In Shizuishan, detected 35 diarrhea stool samples including eight samples (22.86%) of *E. coli* K99; 10 samples of BRV (28.57%); two samples of BCoV (5.71%); one sample of BToV (2.86%); one sample of BKoV (2.86%); 14 samples of *Cryptosporidium* (40.00%).

The Chi-square test showed that the main epidemic pathogen causing diarrhea in Shizuishan calves was BRV ($p < 0.05$). Although *Cryptosporidium* (40.00%) and *E. coli* K99 (22.86%) had higher detection rates in diarrhea stool samples, there was no significant difference between them and normal stool samples. In

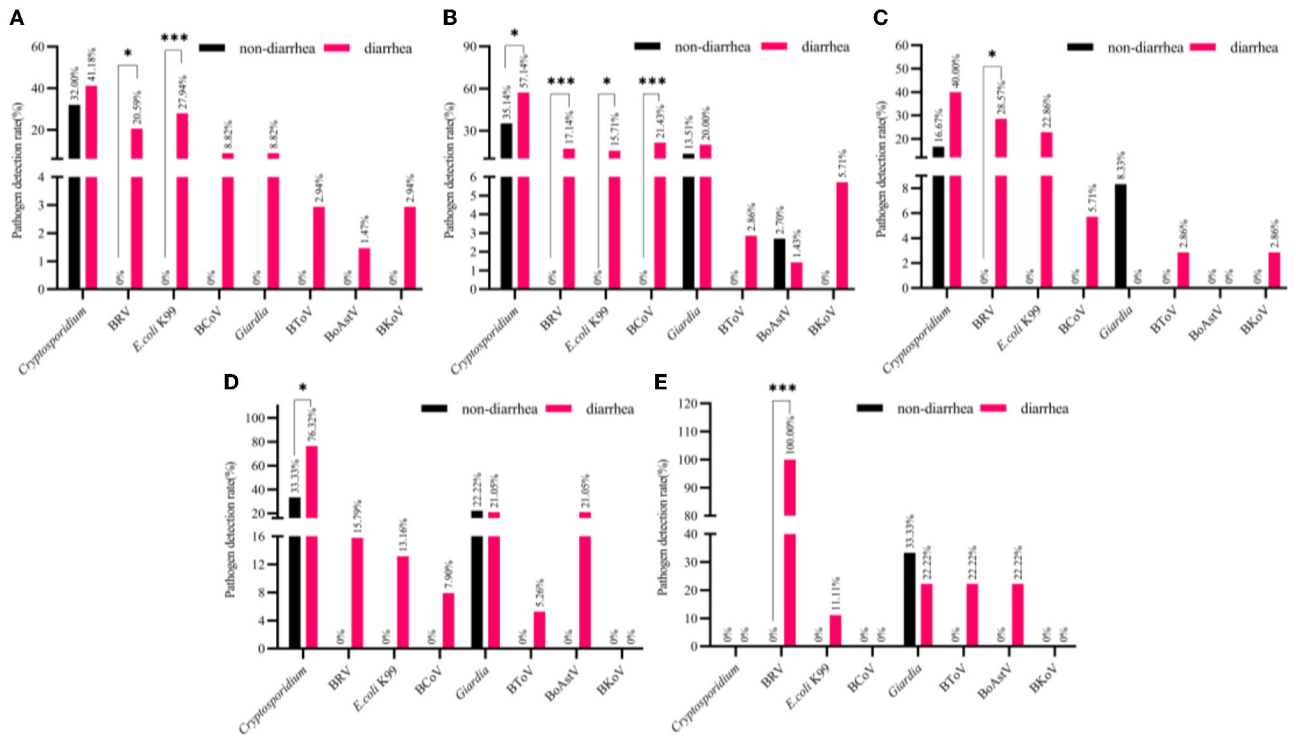


FIGURE 3
The detection rate of normal and diarrhea samples in different cities [**(A)** Yinchuan, **(B)** Wuzhong, **(C)** Shizuishan, **(D)** Zhongwei, **(E)** Guyuan]. The number of pathogen detection and non-detection in normal stool samples with the number of pathogen detection and non-detection in diarrhea stool samples in different cities were counted for chi-square test (mean ± standard deviation) $^{***}p < 0.01$, $^{*}p < 0.05$.

particular, *Giardia* was not detected in diarrhea stool samples, but its detection rate in normal stool samples (8.33%) was second only to *Cryptosporidium* (16.67%). The results are detailed in Figure 3C.

A total of 38 stool samples with diarrhea were detected in Zhongwei: five samples (13.16%) of *E. coli* K99; four samples of BRV (15.79%); three samples of BCoV (7.90%); two samples of BToV (5.26%); eight samples of BoAstV (21.05%); *Cryptosporidium* 29 (76.32%) samples; *Giardia* 8 (21.05%) samples; BKoV and *Coccidia* were not detected.

The Chi-square test showed that the main epidemic pathogen causing diarrhea in Zhongwei calves was *Cryptosporidium* ($p < 0.05$), with a detection rate of 76.32%. The detection rates of other pathogens between diarrhea and normal stool samples were showed no significant difference. The detection rate of *Giardia* in normal stool samples (22.22%) was even higher than that in diarrhea stool samples (21.05%). The results are detailed in Figure 3D.

In Guyuan, a total of nine diarrhea stool samples were detected in *E. coli* K99 in 1 (11.11%); BRV in 9 (100%); BToV in 2 (22.22%); BoAstV in 2 (22.22%); *Giardia* in 2 (22.22%).

The Chi-square test showed that the main epidemic pathogen causing diarrhea in Guyuan calves was BRV ($p < 0.01$). Other pathogens were not significantly different. The detection rate of Giardia in normal stool samples (33.33%) was higher than that in diarrhea stool samples (22.22%), which was similar to the detection of Giardia in Zhongwei and Shizuishan. The results are detailed in Figure 3E.

The detection of pathogen in different seasons

The Chi-square test was performed on the number of cattle farms collected in different seasons and the pathogen detection rate of diarrhea fecal samples in each cattle farm. The correlation between the four main pathogens with the significant difference in detection rate in each season and diarrheal calves was analyzed.

The results showed that the dominant pathogens of diarrhea in spring in Ningxia were BCoV (30.50%), *E. coli* K99 (27.98%), BRV (27.05%) and *Cryptosporidium* (25.38%). In summer, the dominant pathogens of diarrhea were *Cryptosporidium* (59.63%), BRV (35.56%), *E. coli* K99 (31.44%) and BCoV (13.39%). In autumn, the dominant pathogens of diarrhea were *Cryptosporidium* (63.57%), BRV (29.61%), BCoV (19.55%) and *E. coli* K99 (15.56%). In winter, the dominant pathogens of diarrhea were *Cryptosporidium* (75.04%), BRV (53.57%), *E. coli* K99 (16.70%) and BCoV (13.39%). The detail results are illustrated in Figure 4.

After the chi-square test of the entire Ningxia, the pathogens of calf diarrhea that were prevalent in each season have been obtained. Yinchuan and Wuzhong are the concentrated breeding areas of cattle in Ningxia. Analyzing the correlation between the significant pathogens in Yinchuan and Wuzhong in each season and diarrheal calves is more important. Based on the detection rate of different pathogens in the cattle farms of Yinchuan and Wuzhong in different seasons, the average detection rate of pathogens in each cattle farm was calculated, and the epidemic diarrhea pathogens in Yinchuan and Wuzhong in different seasons were obtained.

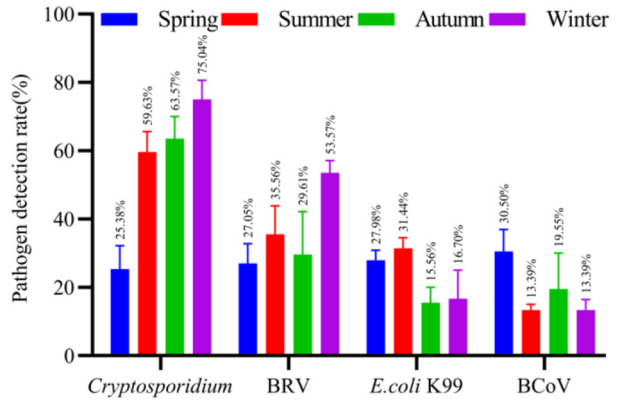


FIGURE 4
The detection rate of normal and diarrhea samples in different seasons. The number of cattle farms in Ningxia where samples were collected at different seasons, and the detection rates of *Cryptosporidium*, BRV, *E. coli* K99, and BCoV in each cattle farm were counted.

In Yinchuan, the dominant pathogens of diarrhea in spring were BRV (39.55%), *E. coli* K99 (36.82%), *Cryptosporidium* (19.55%) and BCoV (4.55%). In summer, the dominant diarrhea pathogens were *Cryptosporidium* (53.46%), *E. coli* K99 (30.39%), BRV (10.00%), and BCoV was not detected. In autumn, the dominant diarrhea pathogens were *Cryptosporidium* (18.18%), BRV (9.09%), BCoV (9.09%), and *E. coli* K99 was not detected. In winter, the dominant diarrhea pathogens were *Cryptosporidium* (92.86%), BRV (28.57%), *E. coli* K99 (16.67%), BCoV (7.15%). The results are detailed in Figure 5A.

In Wuzhong, the dominant pathogens of diarrhea in spring were *Cryptosporidium* (45.00%), BCoV (36.67%), BRV (8.34%), and *E. coli* K99 was not detected. In summer, the dominant diarrhea pathogens were *Cryptosporidium* (81.62%), *E. coli* K99 (21.51%), BCoV (16.45%), BRV (6.03%). In autumn, the dominant diarrhea pathogens were *Cryptosporidium* (70.00%), BCoV (30.00%), BRV (20.00%), *E. coli* K99 (20.00%). In winter, the dominant diarrhea pathogens were BRV (38.33%), BCoV (33.33%), *Cryptosporidium* and *E. coli* K99 were not detected. The results are detailed in Figure 5B.

Distribution of different pathogens in different ages

The earliest onset time and the common age of nine pathogens were illustrated in Figure 6. The earliest onset age of *Cryptosporidium* was 4 days, and the frequent onset age was 5–18 days. The earliest onset age of BRV was 4 days, and the frequent onset age was 7–30 days. The earliest onset age of *E. coli* K99 was 1 day and the common onset age was 8–15 days The earliest onset age of *Giardia* was 7 days, and the most frequent age was 11–30 days. The earliest onset age of BCoV was 2 days, and the most frequent age was 9–26 days. The earliest onset age of BoAstV was 8 days, and the most frequent age was 8-30 days. The earliest onset age of BToV was 8 days, and the most frequent age was 8–44 days. The earliest onset age of BKoV was 10 days, and the most frequent age was 10–26 days. The earliest onset age of *Coccidia* was 27 days.

Relationship between main diarrhea pathogens and river distribution in Ningxia

Ningxia is a province through which the Yellow River flows, with a length of about 397 km. There are two other tributaries, the Qingshui River and the Kushui River. Among the 23 large-scale cattle farms in this study, 18 cattle farms were close to the river, and the average number of *Cryptosporidium* detected per farm was 7.22, of which 5 cattle farms detected *Cryptosporidium* number $\geq$10. Among the five cattle farms where no *Cryptosporidium* were detected and where *E. coli* K99, BRV, and BCoV were the main diarrhea pathogens, three cattle farms were not surrounded by a river and one cattle farm was relatively far from a river.

This suggests *Cryptosporidium* is the main diarrhea pathogen in cattle farms, <500 m from the water source. However, the detection rate of *Cryptosporidium* was positively correlated with the distance from cattle farms to rivers, but not significant ($r = 0.1941$), while the detection rates of *E.coli* K99, BRV, and BCoV were not correlated with the distance from cattle farms to rivers. The specific analysis results are detailed in Figure 7.

Discussion

Wuzhong is the city with the most types of pathogens and the highest average detection rate, followed by Yinchuan. Because the etiology of calf diarrhea is more complex, in addition to other environmental factors, it is predominantly caused by pathogens [viruses (31), bacteria (1), parasites (32)], especially *Cryptosporidium*, which is principally transmitted by fecal-oral transmission (33). Therefore, calf density is one of the important factors affecting its transmission rate, and Wuzhong, Yinchuan, and some counties in Shizuishan and Zhongwei are the location of cattle breeding areas in Ningxia, and the density of calf herds is extremely high more than other cities. Consistently, like the results reported in other studies, *Cryptosporidium* is an important cause of diarrhea in Ningxia calves (33–35). In this study, a total of 315 stool samples were collected from all five cities in Ningxia, and 137 stool samples (43.49%) were positive for *Cryptosporidium*, including diarrhea samples (50.46%) and normal samples (27.37%). In 2015, researchers reported on *Cryptosporidium* infection in Ningxia and Gansu (35), 150 positive samples (5.09%) were detected in 2,945 stools in both diarrhea and normal calves. The detection rate of our study is significantly higher than 5.09%, which suggests that the infection rate of *Cryptosporidium* in Ningxia is rising year by year. Since December 2011, the detection rate of *Cryptosporidium* in Ningxia has shown a significant increase, from 1.68% (23/1,366) (33) to 50.46% (111/220). The infection of *Cryptosporidium* in calves with diarrhea and normal calves also coexist in this study, which is consistent with the results of the above studies.

At present, the treatment measures for *Cryptosporidium* are only preventive, and there is no effective commercial vaccine on the market to prevent long-term infection in cattle. The increased prevalence is one of the serious problems faced by researchers.

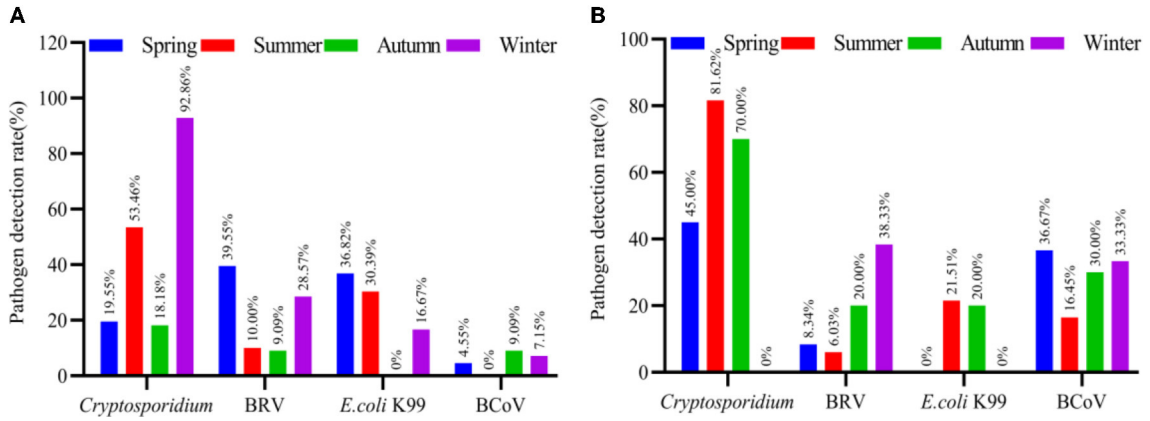


FIGURE 5
The detection rate of normal and diarrhea samples in different seasons [**(A)** Yinchuan, **(B)** Wuzhong]. The number of cattle farms in Yinchuan and Wuzhong, where samples were collected at different seasons, and the detection rates of *Cryptosporidium*, BRV, *E. coli* K99, and BCoV in each cattle farm were counted.

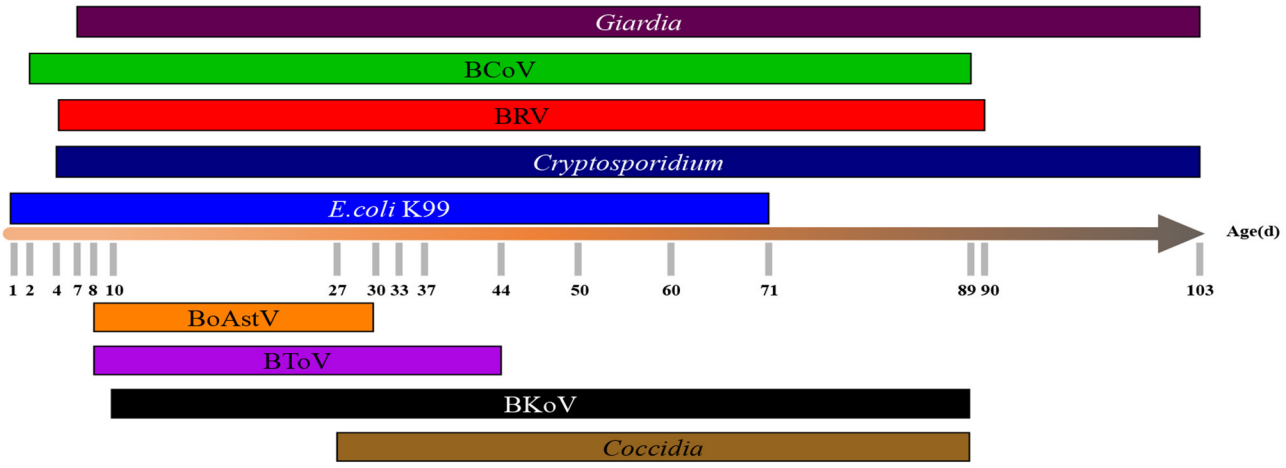


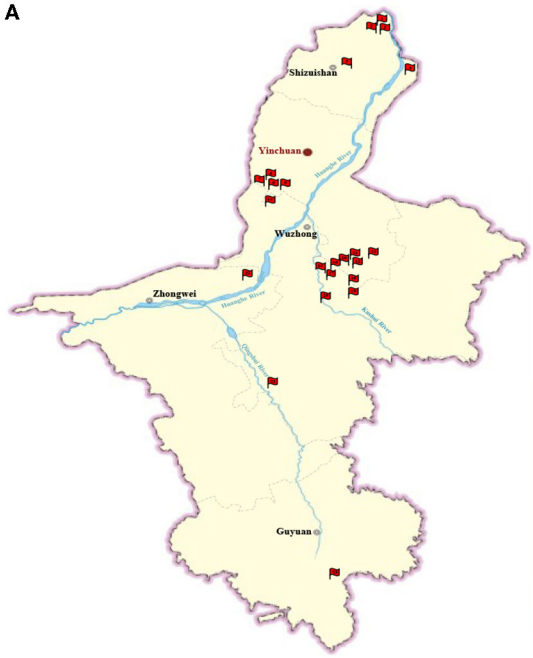
FIGURE 6
The distribution of different pathogens in different ages. The distribution of 9 pathogens in calves of different ages (1–103 d) was counted.

Therefore, it is important to take care of deworming cattle in all growth stages and pay attention to biological safety measures.

BRV is the main pathogen that causes calf diarrhea worldwide. It has been also reported in many regions of China. Rotaviruses are a major causative pathogen of diarrhea in humans and animals, involving the deaths of 200,000 children in developing countries and causing economic losses in the livestock industry globally. In this study, the detection rate of BRV in Ningxia from 2021 to 2022 (23.18 %) was lower than the average detection rate of Ningxia over the years (32%), which was lower than the pooled prevalence of BRV in China 46% (6,635/10,677) (36). This is greatly related to the fact that the Ningxia agricultural department pays more attention to the impact of viruses on the cattle industry.

Compared with *Cryptosporidium* and BRV, the infection rates of *E. coli* K99 in Ningxia were relatively low. However, compared with other pathogens, *E. coli* K99 and other pathogenic *Escherichia coli* are still important pathogens causing calf diarrhea. The detection rate of BoAstV in Zhongwei (21.05%) was significantly higher than that in other cities, but it was not the main cause of diarrhea in Zhongwei calves, the reason may be that the BoAstV detected in this study was neurotype rather than diarrhea type. Evolutionary analyses showed that astrovirus strains from bovine brain tissue were closely related to astrovirus strains from humans, pigs, sheep and other animals with neurological symptoms, indicating that cross-species transmission may occur.

To date, *Cryptosporidium*, *E. coli* K99, BRV and BCoV have been identified as important pathogens prevalent in calf diarrhea in China. In addition, previous studies have demonstrated that BRV can be transmitted to humans directly or through recombination during the evolution of the strain and *Cryptosporidium* and *E. coli* K99, and is typical zoonosis (37). Thus, the in-depth investigation of the above calf diarrhea pathogens is the basis for the prevention and treatment of calf diarrhea, and how to avoid the mixed infection caused by multiple pathogens is of clinical significance. Thus, more efforts should be taken to block the spread of these pathogens in cattle farms and reduce the external factors leading



City	Farm	The distance from farm to river(m)	*Cryptosporidium*	BRV	*E.coli* K99	BCoV
Shizuishan	1.Xiannong	2100	4	0	2	0
	2.Baoma	3100	6	4	0	0
	3.Yinongjinhe	900	6	2	0	1
	4.Ruixiangyuan	2100	0	3	6	1
	5.Chuangwei	-	0	1	0	0
Yinchuan	6.Pingjibao Sixth Dairy Farm	1100	3	1	0	1
	7.Yitai	-	2	2	3	2
	8.Benwang	292	1	2	3	1
	9.Rongjun	10	10	0	2	1
	10.Zerui	10	1	4	7	0
	11.Xingyuanda	2600	1	0	1	0
	12.Baitugang 1st Dairy Farm	954	8	5	0	1
	13.Jiuniu	-	6	0	1	0
Wuzhong	14.Kangsheng	449	13	3	4	3
	15.Wuli	720	2	0	0	1
	16.Anyixin	1200	0	0	0	0
	17.Shangle	459	13	3	2	2
	18.Bencheng	2300	7	2	2	3
	19.Youmuyuan	-	0	3	0	4
	20.Youran	190	15	1	3	0
	21.Yiyang	337	2	0	0	2
Zhongwei	22.Xingken	6300	32	6	5	3
Guyuan	23.Liupanshan	-	0	9	1	0
	Total		132	51	42	26

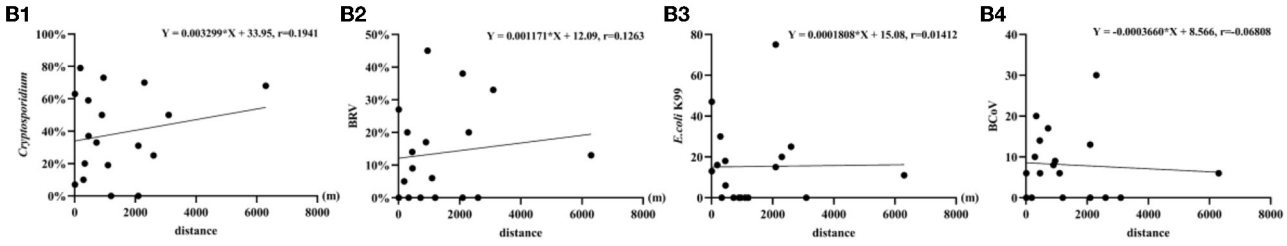


FIGURE 7
The correlation analysis of main pathogens causing calf diarrhea and river distribution. **(A)** The number of *Cryptosporidium*, BRV, *E. coli* K99, and BCoV detected in cattle farms and distribution of sampling cattle farms and rivers. **(B1)** The correlation between the *Cryptosporidium* detection rate and the distance from cattle farm to river. **(B2)** The correlation between the BRV detection rate and the distance from cattle farm to river. **(B3)** The correlation between the *E.coli* K99 detection rate and the distance from cattle farm to river. **(B4)** The correlation between the BCoV detection rate and the distance from cattle farm to river.

to calf diarrhea. In total, it is possible to reduce the incidence of calf diarrhea.

The area around the reiver is a high-frequency area for parasite reproduction and transmission, and many parasites, including *Cryptosporidium*, can be transmitted through water (38, 39). *Cryptosporidium* in its oocyst stage can remain infectious for many months under cool, moist conditions such as rivers, lakes and ponds (40), and in a relatively dry environment, it is more suitable for the growth of viruses and bacteria (24, 41). The distribution of calf diarrhea pathogens in Ningxia also showed similar characteristics in this study, and how to prevent the spread of the pathogen due to geographic environmental factors is one of the issues the researchers have been facing.

Conclusion

In this study, *Cryptosporidium* can be detected in both diarrheal calves and normal calves, and other pathogens are a mixed infection of two or more pathogens in the same or different calves. Together, *Cryptosporidium*, BRV, *E. coli* K99 and BCoV are the main pathogens causing calf diarrhea in Ningxia, the remaining four pathogens are mainly infected in the form of mixed infection.

From June 2021 to May 2022, the main pathogens causing calf diarrhea in Yinchuan were *E. coli* K99 and BRV; the main pathogens causing calf diarrhea in Wuzhong are *Cryptosporidium*, BCoV, BRV and *E. coli* K99; BRV was the main pathogen causing calf diarrhea in Shizuishan; *Cryptosporidium* was the main pathogen causing calf diarrhea in Zhongwei; BRV was the main pathogen causing calf diarrhea in Guyuan.

Different seasons had a more obvious effect on the detection rate of calf diarrhea-related pathogens. In addition, the rivers had an effect on the detection rate of *Cryptosporidium*. In conclusion, the distribution of diarrhea pathogens in Ningxia calves is associated with geographical and environmental factors.

Data availability statement

The original contributions presented in the study are included in the article/supplementary

material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was reviewed and approved by Executive Committee of Laboratory Animal Management and Ethics Inspection of Northwest A&F University, Xianyang, China. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

KG, XK, and HG designed the experiments. DW, LZ, and WD carried out the experiments. HG and CL collected samples. DW wrote the manuscript. XZ and YL contributed to data analysis and helped complete the experiments. All authors discussed the results and commented on the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Molecular detection and characterization of *Anaplasma marginale* infecting cattle, buffalo, and camel populations in southern Egypt

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Anaplasmosis is a severe tickborne disease of ruminants caused by *Anaplasma marginale*. *A. marginale* is distributed worldwide and attacks erythrocytes, resulting in an increased body temperature, anemia, jaundice, abortion, and, in some cases, death. Animals infected with this pathogen become lifelong carriers. In this study, we aimed to detect and characterize *A. marginale* isolated from cattle, buffalo, and camel populations using novel molecular techniques in southern Egypt. In total, 250 samples (from 100 cattle, 75 water buffaloes, and 75 camels) were analyzed by PCR for the presence of *Anaplasmataceae*, specifically *A. marginale*. The animals varied in breed, age, and gender, with most showing no signs of severe disease. By species, *A. marginale* was found in 61 out of 100 (61%) cattle, 9 out of 75 (12%) buffaloes, and only 5 out of 75 (6.66%) camels. All *A. marginale*-positive samples were examined for the heat-shock protein *groEL* gene and, additionally, for *major surface protein 4* (*msp4*) and *major surface protein 5* (*msp5*) genes to enhance specificity. Phylogenetic analysis of *A. marginale* targeted three genes (*groEL*, *msp4*, and *msp5*). This study provides the first report on using three genes for *A. marginale* detection in *Camelus dromedarius* in southern Egypt and generated new phylogenetic data for *A. marginale* infections in camels. *A. marginale infection* is endemic in different animal species in southern Egypt. Screening herds for *A. marginale* is recommended even when the signs of anaplasmosis are absent.

KEYWORDS

***Anaplasma marginale*, cattle, buffalo, camel, Egypt, PCR, sequencing**

1. Introduction

Tickborne diseases are a serious challenge to global health. In Egypt alone, they pose a significant threat to animal health, in particular to local exotic and crossbred cattle and buffalo, thus potentially undermining the livelihoods of their owners (1). *Anaplasma* species are the most common tickborne pathogens in cattle and are endemic across six continents with a high incidence in tropical and subtropical areas of the world (2). The disease they cause is termed anaplasmosis, which is particularly common in ruminants (3). Among *Anaplasma* species (Rickettsiales: Anaplasmataceae), *Anaplasma marginale (A. marginale)* may be the most dangerous.

Anaplasmosis causes progressive hemolytic anemia and significant economic losses in tropical and subtropical areas (4). Ticks are known carriers of *A. marginale*, and approximately 20 tick species have been implicated as vectors of anaplasmosis (5). Bovine anaplasmosis is an economically devastating disease that results in losses to the dairy and beef industries through reduced milk production, weight loss, abortion, jaundice, and sometimes death (6, 7). The disease is mainly spread to cattle by ixodid ticks, but other routes of infection include fly bites and blood-stained objects, such as needles, ear tags, and castration equipment. Placental transmission may feature in the disease's epidemiology in specific areas (8). Fever, anemia, weakness, enlarged lymph nodes, abortion, reduced milk production, and jaundice are signs of anaplasmosis in cattle, and the disease can be fatal in severe cases (9). Cattle recovering from acute infection remain carriers for the rest of their lives and can act as sources of infection for previously naïve livestock populations, triggering endemic infection or epizootics (10).

The camel is a multipurpose animal playing crucial roles in the transport and provision of milk and meat in arid and semi-arid regions of the world. Although camels are hardy animals and can withstand the harsh conditions of dry areas due to their unique, adaptive physiology, their health can be adversely affected by a range of specific diseases (11, 12), including those transmitted by bloodborne parasites. Such diseases can cause anemia, emaciation, and even death in severe cases when camels are infected (13). Camel anaplasmosis has been reported as a subclinical disease in dromedary camels of Tunisia, India, and Saudi Arabia (14).

Anaplasma species are longevous microorganisms, potentially surviving in hosts for months or years, and the consequences of this phenomenon include increased transmission and the occurrence of new anaplasmosis outbreaks (15). Control measures include frequent surveillance, prompt treatment, and eradication of arthropod vectors, and their feasibility depends on several variables, including geographic location, husbandry practices, and implementation costs (encompassing items such as the vaccine or antibiotic treatment programs) (16). Variability in vectoring capacity and limited understanding of the tick's immune response (particularly with regard to arthropod–microbe interactions for bacteria) have impeded control efforts (17). Although current knowledge is limited, vaccines against ticks are being developed (18). *A. marginale* infections in cattle and buffalo have previously been recorded in different parts of Egypt (19–23). In this study, we report on *A. marginale* in three governorates in the southern part of Egypt, which we targeted because of the lack of research on *A. marginale* and its host species in this part of the country. Specifically, we applied molecular techniques to detect and characterize *A. marginale* infecting cattle, buffaloes, and camels.

2. Materials and methods

2.1. Study design and research area

This study focused on anaplasmosis infection in local breeds of cattle, buffaloes, and camels of various ages (from 1 to 3 years) and both genders. It was conducted from April 2021 to January 2022, in three southern governorates in Egypt: Sohag, Qena, and Red Sea governates (Figure 1).

2.2. Clinical examination

Animals underwent clinical examinations before blood sample collection. The examination involved identifying age and gender and evaluating body mass index, body temperature, heart rate, respiratory rate, and visible mucous membranes. Some cattle were presented with pale mucous membranes, and respiratory disorders were noted in a small number of animals. All animals were infested with ticks, although buffaloes and camels showed no visible specifically associated clinical manifestations.

2.3. Collection of samples

Samples were collected from animals selected at random. Small flock breeding mainly determines the species of animal raised by farmers in southern Egypt, which imposes some limitations on sample collection in this region. Accordingly, the number of samples in this study was set so as to provide a clear picture of the epidemiology of the relevant diseases in local animal populations. Whole blood samples were collected from the jugular vein of each animal with clean, sterile vacutainer tubes containing heparin for DNA extraction, as a target for PCR amplification. Samples were kept at −20°C until use.

2.4. Detection of control genes and pathogens by PCR

All primers used in this study are listed in Table 1, and the PCR conditions are shown in Table 2. The amplification of bovine *β-actin* for DNA extract was confirmed by amplifying the bovine and camel *β-actin*–encoding genes (housekeeping genes) to ensure that the genomic DNA had been extracted from all samples (24, 25). Negative controls were samples containing nuclease-free water. Electrophoresis of PCR products was performed with 1.5% agarose gel in 1×Tris–acetate–EDTA (TAE) buffer using a Mupid electrophoresis device (Mupid Co., Ltd., Tokyo, Japan), and bands were visualized through a gel documentation system UV device, WUV-M20 (ATTO Co., Ltd., Tokyo, Japan), after being stained with 5 g/ml ethidium bromide in 1×TAE.

2.5. PCR amplification and DNA extraction

For this study, 250 samples (from 100 cattle, 75 water buffaloes, and 75 camels) were analyzed by PCR for the presence of *Anaplasmataceae*, specifically *A. marginale*. The animals varied in breed, age, and gender, with most showing no signs of severe disease when samples were collected using commercial extraction kits (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA). DNA was then extracted from whole blood samples. *A. marginale* was detected by screening using nested PCR

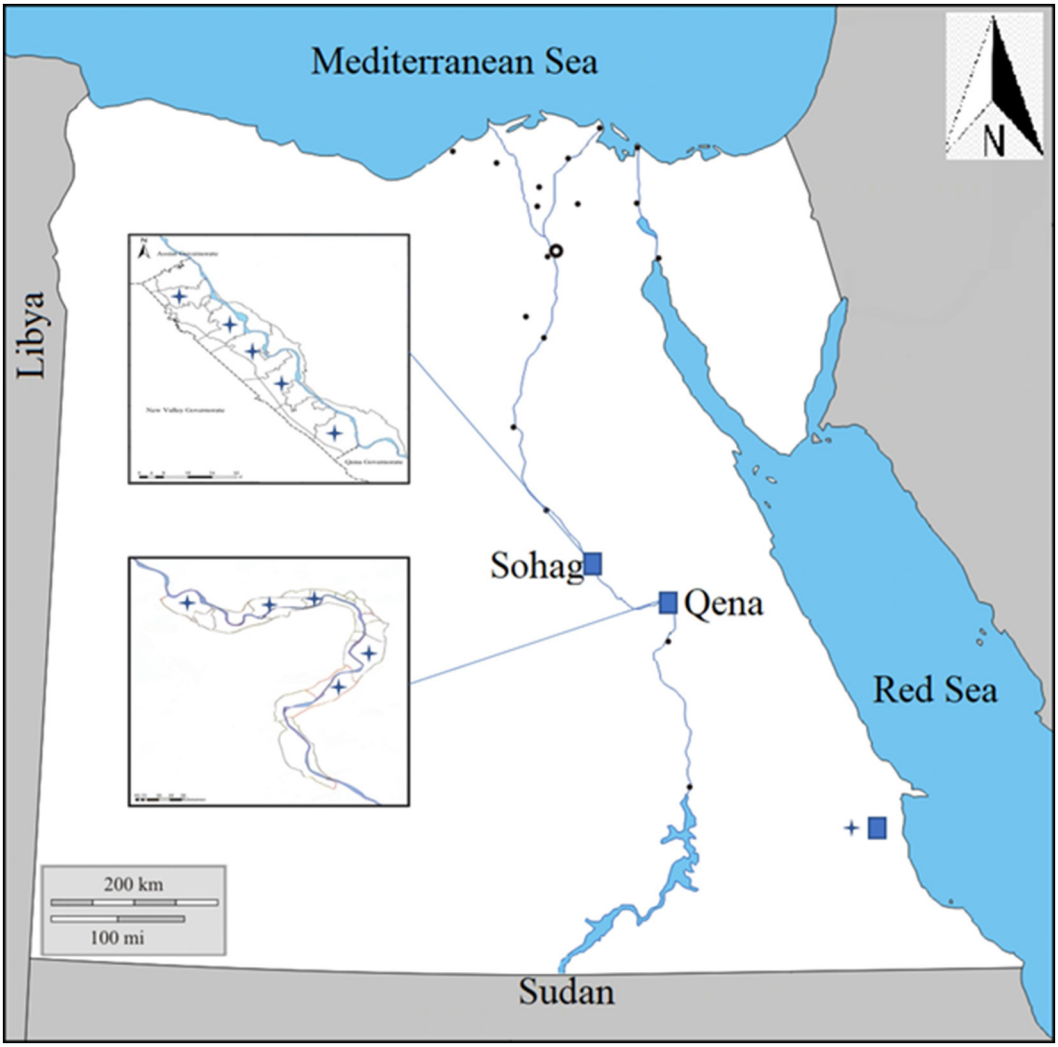

FIGURE 1
Map showing the southern part of Egypt where blood samples were collected from animals in three governorates, Sohag, Qena, and Red Sea.

amplification of the heat-shock protein *groEL* gene using the relevant primers (26). Selected *A. marginale*-positive samples were also subjected to conventional PCR targeting the *msp4* and *msp5* genes (26, 27). The PCR was performed with a total volume of 10 µl, using Tks Gflex DNA Polymerase (TaKaRa), 10 pmol each of the forward and reverse primers, and nuclease-free water. A template (1 µl DNA) was used. The PCR conditions are shown in Table 2. A negative control containing nuclease-free water was added to each PCR. The electrophoresis of the PCR products was performed using 1.5% gel and 1×TAE buffer. The observation was made using a gel documentation system UV device, WUV-M20 (Atto Co., Ltd.), after the gel was stained with 5 µg/ml ethidium bromide in 1×TAE.

2.6. Sequence and data analysis

The selected *A. marginale groEl*, *msp4*, and *msp5* genes were subjected to PCR or 50 µl mixtures for sequence analysis. The amplicons were purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Leicestershire, Duren, Germany), following the manufacturer's protocol. Sequence readings were compared to sequences of reported isolates from a gene bank. A maximum-likelihood phylogenetic tree was constructed using MEGAX software (28), with bootstrap values estimated using 1,000 replicates based on Kimura's two-parameter substitution model (29).

3. Results

3.1. DNA confirmation and identification

A total of 250 blood samples from cattle, buffaloes, and camels were collected from three governorates in southern Egypt. All 250 samples (100%) were confirmed to contain DNA, as they exhibited bands at the expected 227 bp for cattle and buffalo. The expected 438 bp for camels with the *β-actin* gene demonstrated that DNA had been successfully extracted from all samples.

TABLE 1 The primers for the amplification of target fragments of genes of *Anaplasma marginale*.

Organism	Target gene	Primer name	Sequence (5′ → 3′)	Expected size (bp)	References
Blood of cattle and buffaloes	*Bovine β-actin gene*	FBA	CGCACCACCGGCATCGTGAT	227	(24)
		RBA	TCCAGGGCCACGTAGCAGAG		
Blood of camels	*Camel β-actin gene*	FBC	AGAGCTACGAGCTGCCTGAC	438	(25)
		RBC	GGTTGCCTCAATGTCCATCT		
Anaplasma marginale	*groEL*	AMgroES111F1	AGAGCTCGAAGGAAAGAAGTTCATAG	580	(26)
		AMgroEL1557R1	CATGAATACAGCTGCRAGTGACACAG		
		AMgroES67F2	TAATCGCTAAGGAGGCGTAGTC		
		AMgroEL513R2	GTCTTTGGCCCAACTTCCCTTACGCACTG		
Anaplasma marginale	*msp5*	AM-49F	GTGTTCCTGGGGTACTCCTATGTGAACAAG	547	(26)
		AM-595R	AAGCATGTGACCGCTGACAAACTTAAACAG		
Anaplasma marginale	*msp4*	*msp4*F	GGGAGCTCCTATGAATTACAGAGAATTGTT	854	(27)
		*msp4*R	CCGGATCCTTAGCTGAACAGGAATCTTGC		

TABLE 2 PCR conditions for the amplification of target fragments of genes of *Anaplasma marginale*.

Target gene	PCR condition
Bovine β-actin	$\frac{94^\circ C}{5\ min} \rightarrow [\ \frac{94^\circ C}{30\ s} - \frac{65^\circ C}{30\ s} - \frac{72^\circ C}{30s}\ ] 35\times \rightarrow \frac{72^\circ C}{5\ min} \rightarrow 10^\circ C \infty$
Camel β-actin	$\frac{94^\circ C}{5\ min} \rightarrow [\ \frac{94^\circ C}{30\ s} - \frac{63^\circ C}{30\ s} - \frac{72^\circ C}{30\ s}\ ] 35\times \rightarrow \frac{72^\circ C}{5\ min} \rightarrow 10^\circ C \infty$
groEL	1st round : $\frac{95^\circ C}{5\ min} \rightarrow [\ \frac{94^\circ C}{30\ s} - \frac{62^\circ C}{30\ s} - \frac{72^\circ C}{1.5\ min}\ ] 35\times \rightarrow \frac{72^\circ C}{5\ min} \rightarrow 10^\circ C \infty$
	2nd round : $\frac{95^\circ C}{5\ min} \rightarrow [\ \frac{94^\circ C}{30\ s} - \frac{65^\circ C}{30\ s} - \frac{72^\circ C}{1\ min}\ ] 35\times \rightarrow \frac{68^\circ C}{5\ min} \rightarrow 10^\circ C \infty$
msp4	$\frac{94^\circ C}{5\ min} \rightarrow [\ \frac{94^\circ C}{30\ s} - \frac{60^\circ C}{30\ s} - \frac{68^\circ C}{1min}\ ] 35\times \rightarrow \frac{68^\circ C}{7\ min} \rightarrow 10^\circ C \infty$
*msp5**	$\frac{95^\circ C}{5\ min} \rightarrow [\ \frac{95^\circ C}{30\ s} - \frac{74-68^\circ C}{30\ s} - \frac{72^\circ C}{1\ min}\ ] 36\times \rightarrow \frac{72^\circ C}{5\ min} \rightarrow 10^\circ C \infty$

*Annealing with 0.2°C incremental decreases until reaching the final annealing temperature of 68°C.

TABLE 3 Detection of *Anaplasma marginale* in cattle, buffaloes, and camels from southern Egypt based on PCR detection in blood samples.

Species	Number of animals	Number of negative	Number of positive	Percent positive
Cattle	100	39	61	61.00 %
Buffalo	75	66	9	12.00 %
Camel	75	70	5	6.66 %
Total	250	175	75	30.00 %

All samples were then subjected to nested PCR to detect the presence of the *A. marginale groEL* gene. The prevalence of *A. marginale* was 75 out of 250 (30%) samples. By species, 61 out of 100 (61%) cattle were *A. marginale* positive, while 9 out of 75 (12%) buffaloes and only 5 out of 75 (6.66%) camels were *A. marginale* positive (Table 3). All samples positive for the *A. marginale groEL* gene were further examined for two additional genes (*msp4* and *msp5)* to provide an enhanced degree of specificity for the identification of *A. marginale*.

Furthermore, a higher prevalence of *A. marginale* infection was found in Qena than in Sohag and Red Sea governates. We found no sex difference in any species in this study, based on the relative prevalence of *A. marginale* in males and females. Further investigations of risk factors should encompass univariate and multivariate analyses targeting animal and farm levels. Even so, we found a high prevalence (36% infection rate) in young animals (1 year old or less) relative to the adult animals. The breeding system also appears to be associated with the risk of *A. marginale* infection. Individually, bred animals had a lower infection rate than intensively bred animals (25 vs. 33.3%; Table 4).

3.2. Sequence analysis

The *A. marginale* heat-shock protein *groEL* gene and major surface proteins *msp4* and *msp5* genes were sequenced for phylogenetic analysis and genotyping in cattle, buffaloes, and camels from three different governates in southern Egypt. All sequences were also submitted to a gene bank, and the following accession numbers can be used to access them: for the *groEL* gene (cattle: OP081155.1, OP081156.1, and OP081157.1; buffalo: OP081158.1 and OP081159.1; camel: OP081160.1 and OP081161.1); *msp4* gene (cattle: OP142721.1 and OP142722.1; camel: OP142723.1 and OP142724.1; buffalo: OP142725.1 and OP142726.1); and *msp5* gene (cattle: OP142716.1 and OP142717.1; buffalo: OP142718.1 and OP142719.1; camel: OP142720.1). Phylogenetic analysis established the relationships for *A. marginale* with the sequences identified for this study, and various isolates from other countries or other geographic locations in Egypt (Figures 2–4).

A phylogenetic tree was constructed to compare the *groEL* gene for cattle, buffaloes, and camels with amplicons separated

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TABLE 4 *Anaplasma marginale* infection in cattle, buffaloes, and camels in three governorates in southern Egypt.

Factors		Locations						Age						Gender				Breeding system			
		Sohag		Qena		Red See		1 year		2 years		3 years		Male		Female		Individual		Intensive	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Number of positive-testing animals		30	30	40	40	5	10	18	36	30	30	27	27	15	30	60	30	25	25	50	33.3
Number of negative-testing animals		70	70	60	60	45	90	32	64	70	70	73	73	35	70	140	70	75	75	100	66.7
Total number of tested animals		100		100		50		50		100		100		50		200		100		150	

N = Number, % = Percent.

from other reported isolates. We found the following alignment identities: 100% to the Philippines, JQ839014.1 and LC461539.1; Malawi, LC664078.1; China, KX987398.1; Uganda, KY523021.1; 99.79% to Japan, FJ226455.1; and 98.07% to Mozambique, KR492655.1 (Figure 2).

For *msp4* amplicons from this study for buffaloes, OP142725.1 and OP142726.1 were identified by the following alignment identities: 100% with amplicons isolated in Western Cuba MK809382.1 and Cuba MK809389.1; 99.88% with Western Cuba MK809387.1; 99.63% with Thailand MH939155.1; 99.38% with USA AY010253.1; 99.25% with Italy EU436159.1; 99.13% with USA AY127072.1; and 99% with Zimbabwe AY666010.1, India KX989521.1, and Hungary HM063432.1. However, for cattle and camel *msp4* amplicons from this study, cattle OP142721.1 and OP142722.1 and camel OP142723.1 and OP142724.1 did not show 100% identity with any amplicon data in the gene bank. They showed 99.75% identity with Zimbabwe AY666010.1 and Hungary HM063432.1; 99.63% identity with India KX989521.1 and Sudan KU497715.1; 99.50% with USA AY010253.1; 99.38% with USA AY127072.1; 99.25% with Italy EU436159.1; 99.13% with Thailand MH939155.1; and 99.00% with Western Cuba MK809382.1 and Cuba MK809389.1. The phylogenetic tree for the *msp4* gene showed that the amplicons from this study for cattle and camel were clustered in a single branch and closely related to a separate branch for other reported isolates from cattle in Sri Lanka and China (Figure 3).

We compared *msp5* amplicons from this study for cattle OP142716.1, buffalo OP142718.1 and OP142719.1, and camel OP142720.1 with amplicons from other reported isolates and found alignment identities of 100% with Sri Lanka LC467691.1 and China KR047042.1; 99.61% with Egypt LC554225.1 and KU042081.1, Philippines AB704328.1, and Benin KX685369.1; 99.59% with Thailand MK240314.1; 99.42% with Egypt LC554224.1 and KU042080.1 and Kenya KP347554.1; 99.40% with Thailand MK164571.1; and 97.86% with the USA M93392.1 (Figure 4).

4. Discussion

In this study, we addressed a paucity of complete data on *Anaplasma* species in southern Egypt distributed among cattle, buffaloes, and camel populations. *A. marginale* infection may be more common than previously believed, possibly due to misdiagnosis and undetected carrier animals. We suggest that more detailed information on the distribution of anaplasmosis in southern Egypt is urgently required.

Anaplasmosis is a tickborne rickettsial disease that can adversely affect livestock health and performance worldwide (30). Anaplasmosis reportedly incurs an average cost of $793 per head of cattle, 54% of which can be attributed to death, followed by 15% attributable to treatment, 14% to weight loss, 8% to chronic disease, and 9% to abortion (31). Previous studies in Egypt have shown a wide distribution of *A. marginale* in cattle and water buffalo; however, the data are still incomplete for camels. In Egypt, *A. marginale* is the second-most common tickborne disease in cattle (21.2%); the infection rate in buffaloes is 37.5%, and dromedaries have reportedly been infected with *Babesia* (11.0%), *Theileria* (71.8%), and *Anaplasma* species, as

FIGURE 2
Phylogenetic relationships based on the sequences of the *heat-shock protein* (*groEL*) gene from *A. marginale* using the maximum-likelihood method and Kimura's two-parameter model, with branch lengths measured in the number of substitutions per site. The tree is depicted to scale. The percentage of trees in which the connected taxa clustered together is displayed next to the branches. Red, green, and blue circles represent *A. marginale* obtained in the present study.

well as *C. burnetti* (20.8%) and *Rickettsia spp*. (31.9%) (32). For anaplasmosis in Egypt, the highest proportions of seropositive animals have been reported in Gharbia (100%), Suez (83.3%), and Port Said (33.3%), whereas the lowest proportions have been recorded in Sohag (4.7%) and Aswan (5.2%) (23). This study was performed to determine the presence of *A. marginale* in cattle, buffaloes, and camels in southern Egypt. The discovery of high prevalence rates of *Piroplasma* and *Anaplasmataceae* among animals that appeared to be in good health—when considered together with the recent rise in international animal trading—suggests the possibility of new genotypes of infections emerging and re-emerging in Egypt following a spread of pathogens from surrounding endemic countries (33, 34). Buffalo from southern Egypt show lower infection rates than cattle from similar areas, and these results may indicate a natural resistance against *A. marginale* in Egyptian buffalo. Previous studies have also demonstrated that water buffalo may show reduced infectivity and cellular replication for this pathogen, resist natural tick infestation, and have a reduced potential for transmitting tickborne diseases (35). The immune system can protect buffalo against high rickettsia levels and related diseases in their acute phase (36). Furthermore, we found that camels had a lower infection rate than cattle and buffaloes, with only five out of 75 camels (6.66%) positive for *A. marginale*. At least four anaplasma species (*A. marginale*, *A. platys*, *A. phagocytophilum*, and *Candidatus A. camelii*) have been identified as infecting *Camelus dromedarius*. However, infection with *A. marginale* in camel was detected primarily by conventional blood testing with stained blood smears, whereas other *Anaplasma* species in camel were either identified serologically or molecularly (37–40),

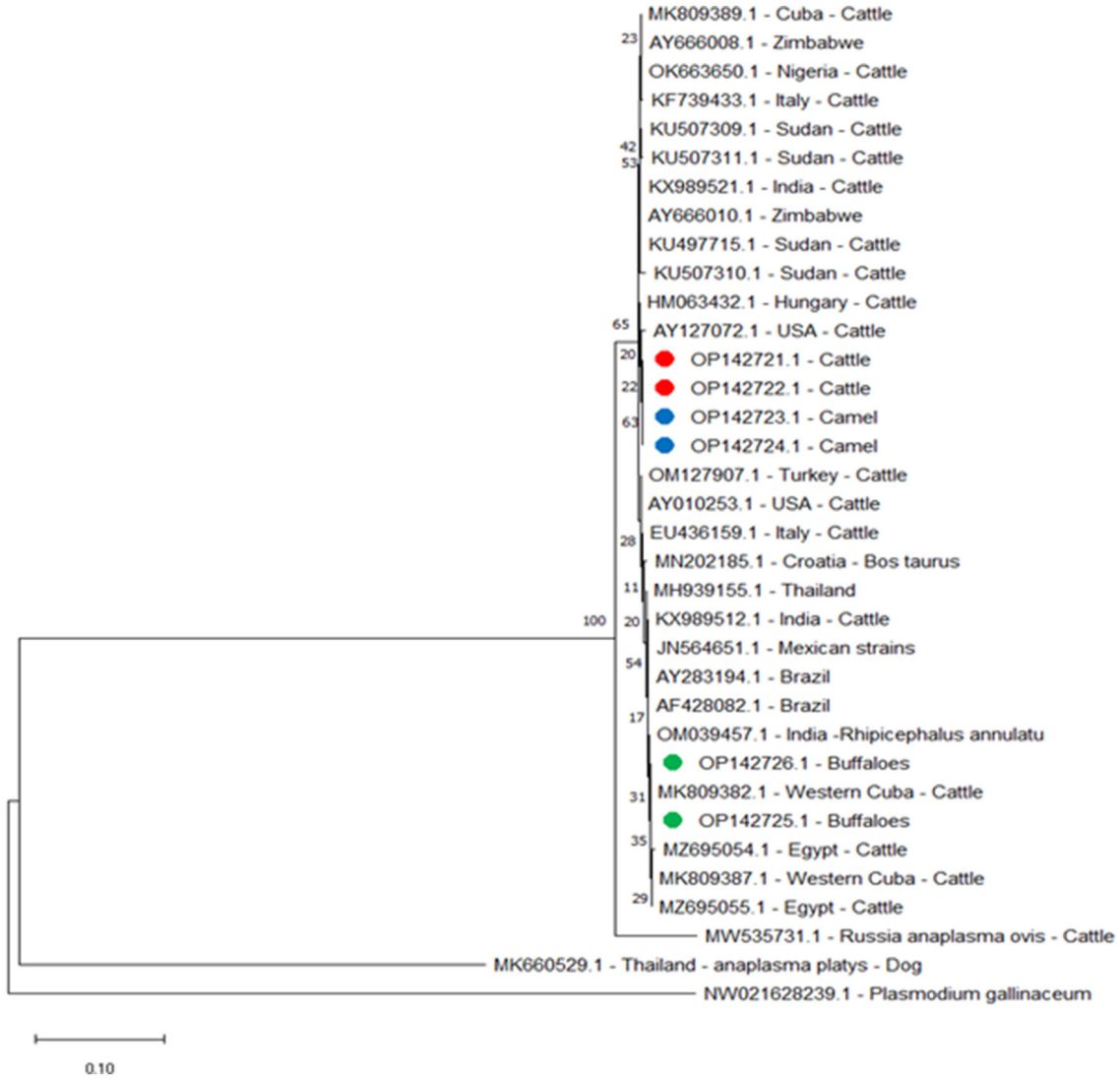

FIGURE 3
Phylogenetic relations of *A. marginale* using the maximum-likelihood method and Kimura's two-parameter model based on *major surface protein 4* (*msp4*) gene sequences. The percentage of trees in which the connected taxa clustered together is displayed next to the branches. Branch lengths are expressed in terms of the number of substitutions per site, and the tree is drawn to scale. Red, green, and blue circles represent *A. marginale* obtained in this study.

using *Anaplasmataceae* 16S rRNA-based amplification, sequencing, and phylogenetic tree construction for *A. phagocytophilum*, *A. platys*, *A. ovis*, and *Candidatus A. camelii* (41). In only one study, in the Riyadh region of Saudi Arabia, have Arabian camels been shown to have *Anaplasma* species, by amplifying the particular *msp5* gene; the pathogen species was determined to be *A. marginale* (42). To the best of our knowledge, this study presents the first report on using molecular methods and phylogenetic analysis to identify *A. marginale* in dromedary camels in southern Egypt.

Based on these epidemiological results and the genetic variation of *A. marginale* detected in loci different from previous studies (43, 44), we conclude that the prevalence and epidemiological characteristics of *A. marginale* infection are closely related to its geographic location.

Major surface protein genes are under selective pressure from the host immune system and play a significant role in the interaction of *Anaplasma* species with host cells (45–47). All *Anaplasma* species studied thus far have orthologs of the immunodominant outer membrane protein *msp4* (46). Both prokaryotes and eukaryotes have a highly conserved housekeeping gene called g*roEL*. *Ehrlichia, Rickettsia*, and *Anaplasma* species are all members of the *Rickettsiales* bacterial family, and this gene has recently been used in phylogenetic analyses of these species (47, 48). Six membrane surface proteins of the initial bodies of this organism (carriers of epitopes B and T) have been characterized. Major surface proteins have been named and identified as 1a, 1b, 2, 3, 4, and 5 (49); these proteins were recognized by neutralizing antibodies, and they have a strong intermolecular relationship in the membranes of the initial bodies, performing essential functions

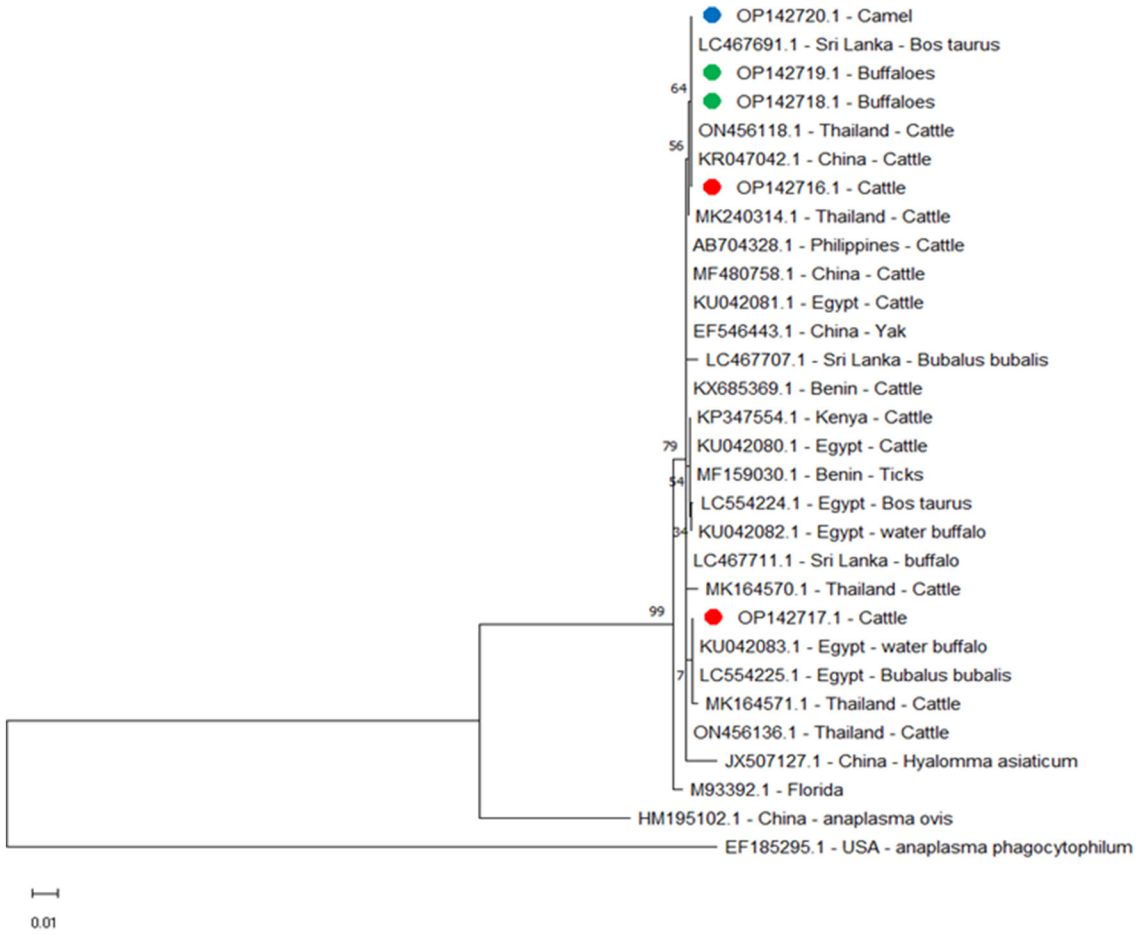

FIGURE 4
Phylogenetic relationships of *A. marginale* using the maximum-likelihood method and Kimura's two-parameter model based on *major surface protein* 5 (*msp5*) gene sequences. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Red, green, and blue circles represent *A. marginale* obtained in this study.

(36). Genes encoding these proteins have been studied, showing their protein products to have a variable polymorphism. They can be represented in the genome by a single copy gene or by forming part of multigenic families (50). All *Anaplasma* species and all examined isolates of *A. marginale* have shown a single copy of the *msp5* gene present in their genomes. This gene is highly conserved, and it is a strong candidate for diagnosing bovine anaplasmosis, since the *msp5* protein it produces has low structural complexity, is similarly conserved, and elicits high antibody titers (51).

Whenever infected cattle are moved, a new genotype of *A. marginale* is imported to their new location, and this genotype may then spread to susceptible cattle either mechanically or biologically. Few genotypic variations are detected in *A. marginale* isolates from places like Australia where cattle movements are rare (52). Dogs were shown to be carriers of ticks that disseminated *A. marginale* infection to cattle herds, and it is thus likely that physical contact between animals could result in tick transmission from one host to another, spreading tickborne diseases between them (53). The close contact between cattle and buffalo, particularly in the individual breeding system, could be a factor in the transmission of *A. marginale* between the different animal species. On the other hand, there is minimal interaction between camels and other animals; however, camels can be transported by the same vehicles as used to transport cattle and buffaloes, and such vehicles may become a path of infection and contribute to the spread of *A. marginale* in animal populations in southern Egypt and elsewhere.

The prevalence of *A. marginale* is known to vary according to environmental conditions, sample site, vector species, host breed, and breeding system (54). According to our research, intensive breeding systems had a higher infection rate than individual breeding systems (33.3 vs. 25%). This may be because there is more animal contact in intensive breeding systems than in individual breeding systems, making it easier for ticks to spread from one animal to another. Management practices differ from farm to farm in the southern area, where most farms house small numbers of co-reared animals because most farmers

implement a multidisciplinary system encompassing pastoral and arable farming. Accordingly, predicting direct effects on disease epidemiology is challenging due to limitations in potential study populations.

Phylogenetic analyses using the *msp4* gene have been used to elucidate the biogeography and evolution of the *Anaplasma* species (46). Phylogenetic analysis based on *msp4* for *A. marginale* showed that the amplicons from this study for cattle and camel cluster in a single branch and have a close relationship with separate branches of other reported animal amplicons. According to one report in 2022, *A. marginale* was detected in camels in southern Egypt using the *msp5* gene; however, that report did not provide any data on phylogenetic analyses of *A. marginale* in camels (55).

The phylogenetic analyses based on *groEL* and *msp5* genes produced very similar results to those on isolates from other locations, possibly due to the unregulated movement of live animals between locations in Egypt for slaughter and marketing. Such local circulation of pathogens should be considered even though the issue of globally circulating tick diseases has gained attention recently with the importation of live animals from other countries to Egypt.

5. Conclusion

The findings of this study indicate that *A. marginale* is highly prevalent in *camels*, cattle, and buffaloes in southern Egypt. The identity of the *A. marginale* was confirmed by amplifying the specific *msp4* and *msp5* genes in phylogenetic analysis, which provided new data for *A. marginale* in southern Egypt. According to obtained results, *A. marginale* infection is endemic in different animal species in southern Egypt. It is the first report using three genes for *A. marginale* in *Camelus dromedarius* in southern Egypt.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository or repositories and accession number(s) can be found in the article or supplementary material.

Ethics statement

The animal study was reviewed and approved by the Faculty of Veterinary Medicine, South Valley University (10/09.02.2021, 53/13.09.2022).

Author contributions

HM and TT: conceptualization, design, experiments, formal analysis, investigation, writing of the original draft, project administration, and funding acquisition. HM, AA, and TT: resources, shared materials, and writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Antibacterial potential of commercial and wild lactic acid bacteria strains isolated from ovine and caprine raw milk against *Mycoplasma agalactiae*

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Introduction: The complexity of fighting contagious agalactia (CA) has raised the necessity of alternative antimicrobial therapies, such as probiotics. Lactic acid bacteria (LAB) are present in the mammary gland of small ruminants and their antimicrobial effect have been previously described against species like *Mycoplasma bovis* but never against *Mycoplasma agalactiae* (Ma). This *in vitro* study aims to evaluate the antimicrobial activity against Ma of ovine and caprine LAB strains and a human commercial probiotic (L2) of *Lactobacillus* spp.

Methods: A total of 63 possible LAB strains were isolated from nine ovine and caprine farms in Spain, three isolates (33B, 248D, and 120B) from the 63 strains were selected, based on their capacity to grow in a specific medium *in vitro*, for an *in vitro* experiment to assess their antimicrobial activity against Ma in Ultra High Temperature (UHT) processed goat milk (GM). A women commercial vaginal probiotic was also included in the study. The inoculum of L2 was prepared at a concentration of 3.24×10^8 CFU/mL and the average concentration of the inoculum of the wild LAB varied from 7.9×10^7 to 8.4×10^8 CFU/mL.

Results: The commercial probiotic L2 significantly reduced the concentration of Ma to 0.000 log CFU/mL ($p<0.001$), strain 33B reduced it from 7.185 to 1.279 log CFU/mL ($p<0.001$), and 120B from 6.825 to 6.466 log CFU/mL ($p<0.05$). Strain 248D presented a bacteriostatic effect in GM. Moreover, the three wild strains and the commercial probiotic produced a significative reduction of the pH ($p<0.001$).

Discussion: This is the first *in vivo* report of the antimicrobial potential of LAB strains against Ma and its interaction. Our results support possible future alternative strategies to antibiotic therapy, previously not contemplated, to fight CA in small ruminants. Further studies are necessary to elucidate the action mechanisms through which these LAB are able to inhibit Ma and to assess the safety of using these strains in possible *in vivo* studies.

KEYWORDS

***Lactobacillus*, *Enterococcus*, mastitis, contagious agalactia, antimicrobial activity, *Mycoplasma agalactiae*, probiotic, raw milk**

1. Introduction

Contagious agalactia (CA) is an infectious syndrome with an important socioeconomic impact on the small ruminant dairy sector due to negative effects on milk production, premature culling, lessen growth rates, and the high costs of control measures. It is characterized by a triad of clinical manifestations: mastitis, arthritis, and keratoconjunctivitis, but can occasionally affect the reproductive and respiratory tract (1–3). It is a multi-etiological syndrome as four different species from the genus *Mycoplasma* are involved in goats: *Mycoplasma agalactiae* (Ma), *Mycoplasma mycoides* subsp. *capri*, *Mycoplasma capricolum* subsp. *capricolum*, and *Mycoplasma putrefaciens*. Ma is considered as the main etiological agent that affects goats and sheep, as the other three species of mycoplasmas have only been described sporadically as the cause of the disease in the ovine specie (4, 5).

Nowadays, the fight against CA is based on vaccination and antibiotic therapy but the absence of a satisfactory strategy causes difficulties to eradicate CA in endemic regions. In Spain, a national voluntary program based on an accurate diagnosis and the control of the disease has been put in place (6). On one hand, vaccination against CA has its limitation; while commercial vaccines can reduce symptoms and excretion (3), it does not prevent shedding in milk (7) and therefore the carrier state persists (8). Different explanations have been suggested for the lack of an efficient vaccination such as the complex etiology in goats, the high plasticity of the genome of circulating strains or their capacity to evade the immune system (3). In this sense, the development of vaccines that can prevent satisfactorily the infection in flocks or the entrance in areas free of CA does not seem to be a short- and medium-term achievement.

On the other hand, antimicrobial therapy can improve the animals' health, but it does not eliminate the pathogen (8). It is assumed that antimicrobial agents can reduce the bacterial excretion and clinical symptoms. Nevertheless, the use of antimicrobial agents can generate antimicrobial resistances (AMR), which can compromise the effectiveness of the antimicrobial therapy (3). Indeed, several studies have reported a reduction in the antibiotic susceptibilities of the mycoplasma species associated with CA in different countries (9–16).

In this context surrounding the control and prevention of CA, the necessity to explore alternative therapies, such as the use of probiotics in recent years in people and animals, has emerged. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (17). Lactic acid bacteria (LAB) offer various advantages as potential probiotics and can be considered as alternatives to antibiotics (18). They are safe microorganisms able to produce different compounds such as bacteriocins, organic acids as lactic acid, hydrogen peroxide, diacetyl, and carbon dioxide that favor the inhibition of pathogenic microorganisms. Lactic acid bacteria are Gram-positive bacteria, they can be found in the microbiota of various anatomical locations such as the oral cavity, the skin, the gastro-intestinal tract, and the reproductive tract (19–23). Their presence in the raw milk of small ruminants is well known (24), and some strains have been tested *in vitro* for their potential probiotic characteristics (25, 26). As far as we know, LAB isolated in small ruminants have never been challenged against pathogens belonging to *Mycoplasma* spp.

In a previous study, a first dose of a commercial vaginal probiotic for women "L1" was intravaginally inoculated in ewes in order to prevent the vaginosis produced by the use of intravaginal devices. This study reported the capacity of L1 to reduce the vaginal neutrophilia produced by estrus-synchronization sponges without altering the animal health status (27). In addition, a higher dose "L2" of this commercial probiotic has been tested *in vitro* against *Mycoplasma bovis* (Mb) in bovine semen and cervical mucus and showed antimicrobial activity against the pathogen. This antimicrobial activity of *Lactobacillus* spp. was associated to their capacity of acidifying the medium (28, 29). In this sense, the *in vitro* sensitivity of Ma to acid pH has been reported in diluted semen of bucks (30). *Mycoplasma bovis* shares 99% of its genome with Ma (31) and both belong to the hominis group, sharing relevant similarities of intrinsic AMR and therefore control measures (32, 33).

The aim of this microbiological study was to evaluate the *in vitro* antimicrobial potential of lactic acid bacteria, isolated from ovine and caprine raw milk, against *Mycoplasma agalactiae* and compare it with the efficacity of the commercial probiotic L2 dose. To achieve this objective, the viability of *Mycoplasma agalactiae* and lactic acid bacteria as well as the extracellular pH oscillations were evaluated in commercial goat milk and in a *Mycoplasma* spp. specific culture medium.

2. Materials and methods

The study design included various steps. The first one was the sampling of 72 animals from nine different farms. The second step involved the isolation of LAB from the raw goat and sheep milk obtained in the first step and the evaluation of their *in vitro* growth capacity in a specific *Mycoplasma* culture medium. The third step consisted in the molecular characterization of the selected strains. The final step was the carrying out of the *in vitro* experiment to assess the antibacterial activity of the different LAB against Ma. All the results from the *in vitro* experiment were statistically analyzed *a posteriori*. In addition, we also analyzed the LAB composition of L2 overtime.

2.1. Animals' description and sampling

Possible LAB strains used in this study ($n = 63$) belong to a collection of the ProVaginBIO investigation group of University CEU—Cardenal Herrera in Valencia, Spain and were isolated from raw milk of ovine ($n = 48$) and caprine animals ($n = 24$), including meat and dairy sheep and goats, from nine different farms (six ovine; three caprine) located in different regions of Spain. The characteristics of the different sampled flocks can be seen in Table 1.

One sheep livestock (herd B) suffered from an outbreak of CA a year before the samples were taken, a reduction in milk production and/or mammary atrophy were observed in 18% of the animals. In this same flock, a strain of Ma with an alarming profile in antibiotic susceptibility tests was isolated. For this reason, antimicrobial therapy was not used. Another flock (herd I) manifested a clinical outbreak during sampling characterized by clinical mastitis, low milk production, and arthritis in kids. In this case, a treatment with tetracyclines was being used in animals showing clinical signs. The use of antibiotics in the other herds was anecdotical.

Prior to the samples collection, a physical examination of the udder was performed through external observation and palpation to rule out the presence of clinical mastitis. A California Mastitis Test (KerbaTEST,

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TABLE 1 Characteristics of the different sampled livestock and the selected LAB strains.

Herd	Specie	Breed	Province	Aptitude	G	NIS	NPS	SS	OD	C
A	Caprine	Murciano-Granadina	Castellón	Dairy	No	22	4	33B	0.336	8.4×10^8
B	Ovine	Manchega	Albacete	Meat	Yes	14	8	120B	0.288	3.2×10^8
C	Ovine	Manchega	Albacete	Dairy	Yes	6	0	-	-	-
D	Ovine	Lacaune	Castellón	Dairy	No	4	0	-	-	-
E	Caprine	Negra-Serrana	Valencia	Meat	Yes	2	1	-	-	-
F	Ovine	Guirra	Valencia	Meat	Yes	5	3	248D	0.131	7.9×10^7
G	Ovine	Lacaune	Alicante	Dairy	No	4	0	-	-	-
H	Ovine	Segureña	Jaén	Meat	Yes	0	-	-	-	-
I	Caprine	Murciano-Granadina/Malagueña	Albacete	Dairy	No	6	2	-	-	-

G, grazing; NIS, no. of isolated strains; NPS, no. of potential strains for the experiment; SS, selected strain for the experiment; OD, optical density after 20 h incubation; and C, concentration in CFU/mL after 20 h incubation.

KERBL) was also performed prior to collecting milk samples to ensure the animals were not affected by subclinical mastitis. *A posteriori*, all the milk samples were inoculated in a modified specific medium for mycoplasmas growth (34), Columbia agar with 5% sheep blood (BD™) and MacConkey agar (BD™) (27) to rule out the presence of mastitis.

2.2. Isolation and selection of lactic acid bacteria

The isolation of LAB was carried out by inoculating the raw ovine and caprine milk samples on Man, Rogosa, and Shape (MRS) agar (Scharlau) (35), and LAB colonies were macroscopically characterized depending on their morphology and frozen at −80°C in cryotubes with 500 µL of liquid MRS and 500 µL of glycerol at 50%.

The 63 isolated strains were tested for their growth in the PH medium. Each strain was activated on MRS agar plates, and one colony was incubated in 4 mL of liquid PH medium at 37°C during 20 h at 150 rpm. Dilutions were performed with phosphate buffer saline solution and four different dilutions were plated on MRS agar. The optical density (OD) was also measured at 600 nm. Strains with OD inferior to 0.100 and with a concentration lower than 10^7 CFU/mL were discarded to assure an effective scale up yield for a possible industrial production of the selected strain. A total of 18 strains met with the selection criteria and four strains, each from a different type of animal production (dairy goat, meat goat, dairy sheep, and meat sheep), with the highest concentration (CFU/mL) post 20 h incubation and an additional strain isolated from herd I, which had an ongoing CA outbreak at the time of sampling, were selected for molecular characterization previous to the *in vitro* experiment. The final three LAB selected to be tested *in vitro* against Ma can be found in Table 1.

2.3. Molecular characterization and bacterial identification of wild LAB strains

The selected strains were characterized, before the *in vitro* experiment. They were processed for genomic DNA extraction and identified based on PCR amplification and sequencing of 16S rRNA gene using bacterial universal primers (27F 5′-AGAGTTTGATCC TGGCTCAG and 1492R 5′-GGTT ACCTTGTTA CGACTT). The PCR was performed following the methodology previously described (21). The PCR products were purified, and sequenced and analyzed for sequence homology by BLAST.[1] The sequences were corrected and aligned by ClustalW with Molecular Evolutionary Genetics Analysis (MEGA) 7. Bacterial identification was carried out by comparing the problem sequence with the GenBank database through the Blast application.

TABLE 2 Composition of the experimental conditions.

Condition	Composition
1	GM (1,460 µL) + Ma (40 µL)
2	GM (1,000 µL) + L2 (500 µL)
3	GM (960 µL) + Ma (40 µL) + L2 (500 µL)
4	GM (1,000 µL) + LX (500 µL)
5	GM (960 µL) + Ma (40 µL) + LX (500 µL)
6	PH (1,460 µL) + Ma (40 µL)
7	PH (1,000 µL) + L2 (500 µL)
8	PH (960 µL) + Ma (40 µL) + L2 (500µL)
9	PH (1,000 µL) + LX (500 µL)
10	PH (960 µL) + Ma (40 µL) + LX (500 µL)
11	GM (1,500 µL)
12	PH (1,500 µL)

GM, semi-skimmed UHT goat milk; Ma, *Mycoplasma agalactiae* strain PG2; L2, commercial probiotic inoculum; LX, ovine/caprine lactic acid bacteria inoculum for each selected strain (33B, 248D, and 120B); and PH, specific medium for *Mycoplasma* spp. growth.

2.4. Design of the *in vitro* experiment

Ten experimental conditions (Table 2) were prepared in Eppendorf-type tubes of 1.5 mL capacity following an adaption of a previous protocol (28, 29). An eleventh (C11) and twelfth (C12) microtubes were included as negative controls. Each wild LAB strain

1 http://www.ncbi.nlm.nih.gov/

(LX) was tested in three independent replicates of the experimental conditions. The conditions were incubated for 15 h.

2.4.1. Preparation of *Mycoplasma agalactiae* inoculum

The Ma inoculum was prepared using the reference strain (PG2, NCTC10123) in PH medium with ampicillin and following the protocol previously described (28, 29). The culture was incubated at 37°C during 48 h, then a subculture was realized and incubated 48 h at 37°C again to obtain our inoculum with an approximate concentration of $1 \times 10^{7\text{-}8}$CFU/mL, based on previous inoculations and the infective dose of Ma (30), and calculated as previously described (36).

2.4.2. Preparation of wild ovine/caprine lactic bacteria inoculum

The ovine/caprine LAB inoculum (LX) consisted of the culture of a single colony of each of the selected LAB strains, previously isolated from raw milk, in 4 mL of PH medium without any added antibiotics at 37°C for 20 h. The tubes were then centrifugated at 4,000 rpm for 15 min. The supernatant was discarded, and the precipitate was reconstituted in microtubes of 1.5 mL with 500 µL of PH medium without antibiotics. The average concentration of the inoculum LX varied from 7.9×10^7 to 8.4×10^8 CFU/mL.

2.4.3. Preparation of L2 inoculum

The inoculum of the commercial probiotic (L2) was prepared at a concentration of 3.24×10^8 CFU/mL as previously described (28, 29). A capsule of a commercial probiotic based on a mix of *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus brevis* (NS Femibiotic®, Cinfa) was reconstituted in PH medium.

2.4.4. Determination of *Mycoplasma agalactiae* and lactic acid bacteria viability

Concentrations (CFU/mL) of Ma and LAB were determined after 15 min (T0) and 15 h (T15). The Ma viability was determined with a protocol of serial dilutions previously described (36) using PH broth supplemented with ampicillin for serial dilutions and PH agar supplemented with ampicillin for bacterial counts (34). The LAB viability was determined on MRS agar plates, with dilutions also performed in PH broth. Every dilution was plated in duplicate.

2.4.5. pH measurement

The pH of every condition was measured with a calibrated pH-meter (SensION™ + pH3, Hach, LPV2000.98.0002) at T0 and T15. The electrode was disinfected with detergent, alcohol and sterile distilled water between the measurement of each condition to avoid contamination.

2.5. Statistical analysis of pH, lactic acid bacteria, and *Mycoplasma agalactiae* viability

Counts of Ma and LAB were transformed as log (1 + C), where C was the count obtained (CFU/mL) for each analytical condition and organism. Statistical analysis was performed using a general linear procedure implemented in the program Statistical Analysis System Institute (SAS), following the model: $Y_{ijk} = \mu + S_i + C_j + T_k + CT_{jk} + e_{ijk}$,

TABLE 3 Processing of raw demultiplexed forward and reverse reads.

Step	Methods used
1. Primer trimming	Dada2 (37)
2. Quality filtering	Dada2
3. Denoising	Dada2
4. Pair-end merging	Dada2
5. Phylotype calling	Dada2
6. Phylogeny assessment	Mafft and Fasttree (38, 39) PRINTDATE * MERGEFORMAT

where Y_{ijk} = pH and log CFU/mL of Ma and log CFU/mL of LAB in each strain studied (33B, 120B, and 248D); µ = mean; S_i = sample effect; C_j = effect of analytical conditions; T_k = effect of time; CT_{jk} = effect of the interaction between the analytical condition and time; and e_{ijk} = residual effect.

2.6. Microbial composition of L2 at T0 and T15

A marker-based approach using the 16S ribosomal RNA subunit gene (16SrRNA) was used to confirm the *Lactobacillus* spp. present in L2 and to study their fluctuation in condition 2 (C2) at T0 and T15, condition 3 (C3) at T0 and T15, condition 7 (C7) at T0 and T15, and condition 8 (C8) at T0 and T15.

The composition and structure of the sampled microbial communities was assessed through the amplification and sequencing the V3-V4 variable regions of the 16S rRNA gene. The Illumina Miseq sequencing 300 × 2 approach was used. Amplification was performed after 25 PCR cycles. A negative control of the DNA extraction was included as well as a positive Mock Community control to ensure quality control. Raw demultiplexed forward and reverse reads were processed as shown in the following Table 3 using QIIME2 (40).

Taxonomic assignment of phylotypes was performed using a Bayesian Classifier trained with Silva database version 138 (99% OTUs full-length sequences) (41).

3. Results

3.1. Identification of wild lactic acid bacteria strains

Based on the sequences obtained, strain 33B was identified as *Enterococcus mundtii* (OQ538168), strain 120B as *Enterococcus hirae* (OQ538169) and strain 248D as *Enterococcus hirae* (OQ538170), and the GenBank submission number being SUB12912028. The other two strains that were selected for molecular characterization were both identified as *Staphylococcus aureus* subsp. *aureus* and were therefore not included in the *in vitro* experiment.

3.2. *In vitro* experiment negative controls

The conditions C11 (GM) and C12 (PH) always came back negative on sheep blood agar plates, MRS agar plates and PH agar plates at T0 and T15. The average pH of C11 at T0 and T15 ranged

TABLE 4 Least squares means of pH and log CFU/mL of Ma and LAB by time for the strain 33B.

Condition	Composition	Time	Ma (LOG CFU/mL)[1]	LAB (LOG CFU/mL)[2]	pH[3]
1	GM + Ma	0	7.248^{ab}	-	6.59^{gh}
1	GM + Ma	15	7.793^{ab}	-	6.50^{h}
2	GM + L2	0	-	8.760^{a}	6.22^{j}
2	GM+ L2	15	-	8.743^{a}	4.09^{l}
3	GM + Ma + L2	0	7.083^{b}	8.806^{a}	6.35^{i}
3	GM + Ma + L2	15	0.000^{e}	8.714^{ab}	4.20^{l}
4	GM + 33B	0	-	8.465^{abcd}	6.55^{gh}
4	GM + 33B	15	-	8.217^{cde}	5.29^{k}
5	GM+ Ma + 33B	0	7.185^{ab}	7.675^{g}	6.55^{gh}
5	GM + Ma + 33B	15	1.279^{d}	7.789^{g}	5.34^{k}
6	PH + Ma	0	7.069^{b}	-	7.47^{b}
6	PH + Ma	15	8.015^{a}	-	7.29^{c}
7	PH + L2	0	-	8.595^{abc}	6.79^{f}
7	PH + L2	15	-	8.257^{bcde}	6.95^{de}
8	PH + Ma + L2	0	7.138^{ab}	8.803^{a}	6.81^{f}
8	PH + Ma + L2	15	7.126^{ab}	8.424^{abcd}	6.89^{def}
9	PH + 33B	0	-	8.257^{bcde}	6.86^{ef}
9	PH + 33B	15	-	7.873^{efg}	7.00^{d}
10	PH + Ma + 33B	0	7.111^{ab}	8.059^{def}	6.87^{ef}
10	PH + Ma + 33B	15	2.209^{c}	7.806^{fg}	6.84^{ef}
11	GM	0	-	-	6.62^{gh}
11	GM	15	-	-	6.65^{g}
12	PH	0	-	-	7.48^{b}
12	PH	15	-	-	7.64^{a}

GM, semi-skimmed UHT goat milk; Ma, *Mycoplasma agalactiae* strain PG2; L2, commercial probiotic inoculum; 33B, lactic acid bacteria strain 33B inoculum; PH, specific medium for *Mycoplasma* spp. growth; LAB, lactic acid bacteria; [1]SEM: 0.33; [2]SEM: 0.16; [3]SEM: 0.04.
[a–l]Means in the same column with different superscripts differ significantly ($p < 0.05$).

from 6.61 to 6.69, respectively, and the average pH of C12 at T0 and T15 ranged from 7.51 and 7.68.

3.3. Effects on *Mycoplasma agalactiae* and lactic acid bacteria viability and pH

In the *in vitro* proposed model, and for each LAB strain studied, the condition itself, the time and the interaction between condition and time had a significant effect ($p < 0.001$) on the pH and the log CFU/mL of Ma. The factor condition contributed significantly to the observed log CFU/mL of LAB variation in all the LAB strain studies, while the factors time and the interaction between condition and time contributed significantly for the LAB strain 33B and 120B, and for 248D, respectively.

3.3.1. Strain 33B

Table 4 details the evolution of the pH and the viability of Ma and LAB over time for the experiment with strain 33B. In favorable conditions, condition 1 (C1) and condition 6 (C6), Ma concentration did significantly increase, and the pH showed stable values between T0 and T15. The strain 33B produced a statistically significant decrease of the concentration of Ma in GM [condition 5 (C5)] and PH medium [condition 10 (C10)]. The pH decreased significantly ($p < 0.001$) in GM in presence of the 33B strain [condition 4 (C4) and C5] between T0 and T15, but it did not in PH medium [condition 9 (C9) and C10] although it was statistically significantly lower in C9-C10 compared to C12. No differences were observed between T0 and T15 for the concentration of LAB.

3.3.2. Strain 248D

Table 5 details the evolution of the pH and the viability of Ma and LAB over time for the experiment with strain 248D. In favorable conditions (C1 and C6), Ma concentration significantly increased, and the pH showed stable values between T0 and T15. The concentration of strain LAB 248D significantly increased with the presence of Ma in GM (C5). Although it did not reduce the concentration of Ma in GM (C5), it was able to prevent the proliferation of Ma between T0 and T15 as the concentration of Ma did not increase in C5 and it was significantly lower than C1 at T15. The strain 248D was also able to significantly decrease the pH over time in GM (C4-5) although without the presence of Ma (C4) the pH was significantly lower at T15 compared to C5. On the other hand, in the PH medium the concentration of LAB 248D significantly decreased at T15 (C9-10) and Ma increased significantly at T15 with the presence of the strain 248D (C10). The pH of the PH medium was stable over time although the conditions with LAB (C7-10) had a pH significantly lower compared to C6 and C12.

3.3.3. Strain 120B

Table 6 details the evolution of the pH and the viability of Ma and LAB over time for the experiment with strain 120B. In favorable conditions (C1 and C6), Ma concentration significantly increased, and the pH showed stable values between T0 and T15. In presence of strain 120B, a significant decrease in the concentration of Ma can be observed between T0 and T15 in GM, associated with a significantly reduction of the pH (C5). This was not the case in PH medium (C10), where the concentration of Ma significantly increased at T15 associated with a stability in LAB concentration and pH.

3.3.4. Commercial probiotic (L2)

The commercial inoculum L2 was able to completely inhibit Ma in GM as no colonies were observed at T15 in any of three replicas of the three wild LAB strains (C3 in Tables 4–6). The concentration of LAB was similar at T0 and T15 in every experiment except for strain 248D (C2-3, Table 5) where a significant increase of concentration of LAB was observed at T15 in GM. The pH in GM was significantly reduced in all the experiments (C2-C3, Tables 4–6) when L2 was added. No pH reduction was observed between T0 and T15 in PH medium conditions (C7-8 in Tables 4–6), where L2 is present. Nevertheless, there was a significative difference between the pH of medium PH without any LAB (C6 and C12) and C7 and C8 at T0 (Tables 4–6).

TABLE 5 Least squares means of pH and log CFU/mL of Ma and LAB by time for the strain 248D.

Condition	Composition	Time	Ma (LOG CFU/mL)[1]	LAB (LOG CFU/mL)[2]	pH[3]
1	GM + Ma	0	7.020^{d}	-	6.59^{d}
1	GM + Ma	15	8.030^{a}	-	6.57^{d}
2	GM+ L2	0	-	8.878^{cd}	6.42^{d}
2	GM + L2	15	-	9.276^{a}	4.11^{g}
3	GM + Ma + L2	0	6.928^{de}	8.681^{de}	6.45^{d}
3	GM + Ma + L2	15	0.000^{f}	9.227^{ab}	4.21^{g}
4	GM + 248D	0	-	8.635^{de}	6.51^{d}
4	GM + 248D	15	-	8.800^{cde}	4.82^{f}
5	GM + Ma + 248D	0	6.883^{de}	8.584^{e}	6.54^{d}
5	GM + Ma + 248D	15	6.711^{e}	8.999^{bc}	5.14^{e}
6	PH + Ma	0	6.822^{de}	-	7.47^{ab}
6	PH + Ma	15	7.949^{ab}	-	7.33^{b}
7	PH + L2	0	-	8.663^{def}	6.86^{c}
7	PH + L2	15	-	8.642^{defg}	6.82^{cd}
8	PH + Ma + L2	0	6.834^{de}	8.664^{def}	6.84^{c}
8	PH + Ma + L2	15	7.382^{c}	8.693^{de}	6.79^{cd}
9	PH + 248D	0	-	8.778^{cde}	7.00^{c}
9	PH + 248D	15	-	8.418^{fg}	6.94^{c}
10	PH + Ma + 248D	0	7.070^{d}	8.761^{cde}	6.99^{c}
10	PH + Ma + 248D	15	7.719^{b}	8.388^{g}	6.77^{cd}
11	GM	0	-	-	6.58^{d}
11	GM	15	-	-	6.58^{d}
12	PH	0	-	-	7.52^{ab}
12	PH	15	-	-	7.63^{a}

GM, semi-skimmed UHT goat milk; Ma, *Mycoplasma agalactiae* strain PG2; L2, commercial probiotic inoculum; 248D, lactic acid bacteria strain 248D inoculum; PH, specific medium for *Mycoplasma* spp. growth; LAB, lactic acid bacteria; [1]SEM: 0.10; [2]SEM: 0.09; and [3]SEM: 0.08. $^{a-g}$Means in the same column with different superscripts differ significantly ($p < 0.05$).

TABLE 6 Least squares means of pH and log CFU/mL of Ma and LAB by time for the strain 120B.

Condition	Composition	Time	Ma (LOG CFU/mL)[1]	LAB (LOG CFU/mL)[2]	pH[3]
C1	GM + Ma	0	6.798^{d}	-	6.65^{d}
C1	GM + Ma	15	7.726^{a}	-	6.68^{cd}
C2	GM + L2	0	-	8.713^{abcde}	6.36^{de}
C2	GM + L2	15	-	9.018^{ab}	4.19^{g}
C3	GM + Ma + L2	0	6.774^{d}	9.119^{a}	6.35^{e}
C3	GM + Ma + L2	15	0.000^{f}	8.936^{abc}	4.27^{g}
C4	GM + 120B	0	-	8.576^{bcde}	6.64^{d}
C4	GM + 120B	15	-	8.728^{abcd}	5.50^{f}
C5	GM + Ma + 120B	0	6.825^{d}	8.376^{def}	6.62^{d}
C5	GM + Ma + 120B	15	6.466^{e}	8.490^{cde}	5.43^{f}
C6	PH + Ma	0	6.689^{de}	-	7.57^{a}
C6	PH + Ma	15	7.424^{b}	-	7.71^{a}
C7	PH + L2	0	-	8.614^{bcde}	6.92^{bc}
C7	PH + L2	15	-	8.667^{bcde}	6.90^{bc}
C8	PH + Ma + L2	0	6.928^{cd}	8.789^{abcd}	6.90^{bc}
C8	PH + Ma + L2	15	6.858^{d}	8.523^{cde}	6.93^{bc}
C9	PH + 120B	0	-	8.570^{bcde}	7.07^{b}
C9	PH + 120B	15	-	7.951^{f}	7.08^{b}
C10	PH + Ma + 120B	0	6.838^{d}	8.669^{abcde}	7.04^{b}
C10	PH + Ma + 120B	15	7.154^{c}	8.276^{ef}	7.02^{b}
C11	GM	0	-	-	6.62^{d}
C11	GM	15	-	-	6.83^{bcd}
C12	PH	0	-	-	7.53^{a}
C12	PH	15	-	-	7.78^{a}

GM, semi-skimmed UHT goat milk; Ma, *Mycoplasma agalactiae* strain PG2; L2, commercial probiotic inoculum; 120B, lactic acid bacteria strain 248D inoculum; PH, specific medium for *Mycoplasma* spp. growth; LAB, lactic acid bacteria; [1]SEM: 0.09; [2]SEM: 0.16; [3]SEM: 0.10. $^{a-g}$Means in the same column with different superscripts differ significantly ($p < 0.05$).

3.4. Lactic acid bacteria composition of L2 per condition and time

Metagenomic analysis (Figure 1) revealed that the three LAB species in conditions containing L2 (C2-C3 and C7-C8) were *Lactobacillus crispatus, Lactobacillus gasseri*, and *Lactobacillus brevis*, as described by the manufacturer, at both T0 and T15. *Lactobacillus crispatus* was always the most abundant LAB specie in all the conditions mentioned at T0 and T15, although its relative abundance (RA) decreased at T15 in every condition, with a RA > 50% except at T15 in GM with the presence of Ma (C3). *Lactobacillus gasseri* was the second most abundant of all three species at both times except at T15 in C3. The RA of *L. gasseri* at T15 stayed similar in GM (C2-C3) but increased in PH medium (C7-C8). Finally, *L. brevis* was always the least abundant specie, except in C3 at T15 where it was more abundant than *L. gasseri*. Its RA increased at T15 in every condition apart from C8.

4. Discussion

The present *in vitro* study reports the antimicrobial effect against Ma of a selection of wild LAB isolates from the milk of healthy sheep and goats. These strains were isolated in herds located in the mainland of Spain (Table 1), an area where etiological agents associated with CA have been frequently isolated in ovine and caprine species (4, 42). Contagious agalactia control and prevention represent a challenge due to several factors: presence of asymptomatic carriers, uncontrolled movement of animals, variability in etiology and antigenicity, the limitations of commercially available vaccines and the increasing AMR of mycoplasmas associated with CA (1–3, 10). Our results suggest that the commercial probiotic used in this study, based on a combination of *Lactobacillus* spp., or wild LAB of ovine and caprine origin could have the potential of being used as antimicrobials for the control or prevention of mastitis caused by Ma.

Our work evinces that an important number of bacterial isolations is necessary in various flocks to obtain LAB strains capable of growing

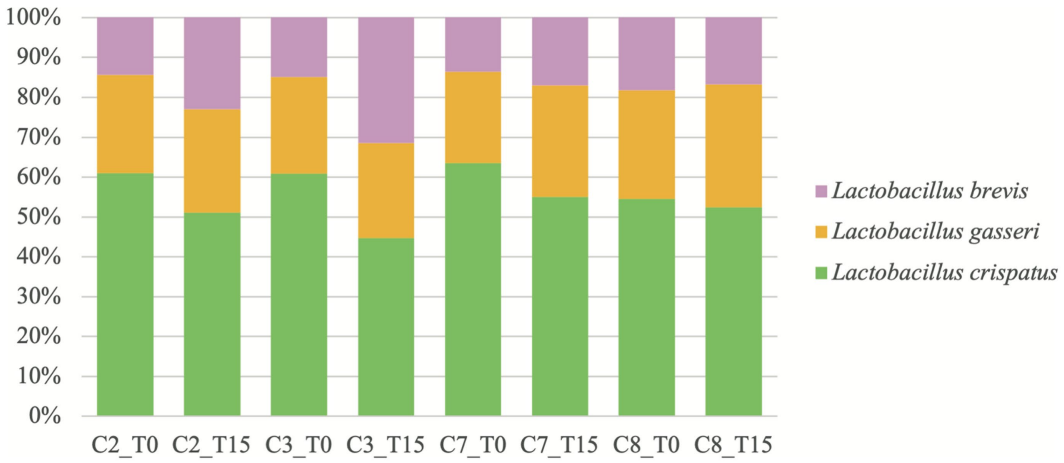

FIGURE 1
Relative abundances, reported as percentages, of *Lactobacillus* spp. over time in conditions where L2 is present. C2: condition 2 with goat milk and L2; C3: condition 3 with goat milk, *Mycoplasma agalactiae* PG2 and L2; C7: condition 7 with PH medium and L2; C8: condition 8 with PH medium, *Mycoplasma agalactiae* PG2 and L2; T0: after 15min incubation; and T15: after 15h incubation.

in a culture medium that allow their *in vitro* testing and demonstrate a possible commercial use (Table 1). All the three wild strains of LAB involved in the *in vitro* experiments were able to inhibit the growth of Ma in GM. Strain 248D had a bacteriostatic effect as it did not significantly decrease the number of Ma at T15, but it did prevent its ease to replicate and increase its concentration in GM at 37°C (Table 5, C5 and C1). Strains 33B and 120B were able to significantly reduce the concentration of Ma at T15 in GM (Tables 4, 6; C5) although the inhibition by 33B was significantly greater than the inhibition produced by 120B ($p < 0.001$).

Lowering the pH is an important feature of LAB as it can inhibit the growth of pathogenic bacteria (43). The acidification of the medium has been suggested to inhibit Ma and *M. mycoides* subsp. *capri*. in diluted semen of bucks as these species are sensitive to pH changes (30). Therefore, one of the causes of the inhibition produced by these LAB may be the drop in the pH of the GM they produced which does not occur when the GM only carries Ma and so the pathogen increases its concentration (C1). All the wild ovine and caprine strains tested in the *in vitro* experiments were able to acidify the GM (Tables 4–6; C4) as there was a significant difference between the GM pH of T0 and T15.

Nevertheless, the strain 33B, identified as *E. mundtii*, was able to inhibit Ma in PH medium (Table 4, C10) with a pH close to neutral and could therefore show better antimicrobial capacity than L2 (Tables 5, 6, C8) in environments where the pH is neutral, and the acidification of the medium is not possible. This suggests that pH acidification may not be the only antimicrobial effect of LAB against Ma and that other antimicrobial mechanisms should be sought.

Probiotics bacteria have several mechanisms of action to inhibit pathogenic bacteria *in vivo*: competing for nutrients, preventing the adhesion of the pathogens, producing inhibitory substances, modulating the host immune response, and reducing the bioavailability of toxins (18). It is unlikely that LAB and Ma compete for the same nutrients given that LAB use glucose to produce lactic acid (44) and Ma cannot ferment glucose unlike other species such as *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capricolum* and *M. putrefasciens* (30). Therefore, we propose the hypothesis that these bacteria could have a greater inhibitory effect against sugar-fermenting mycoplasma species. In the case of Ma, the production of inhibitory substances *in vitro* could be one of the antimicrobial mechanisms used by LAB, in addition to the harmful effect produced by acidification of the extracellular pH, given that the inhibition in PH medium (Table 4, C10) occurred without a medium acidification for the strain 33B.

One of the inhibitory substances produced by LAB are bacteriocins, and raw milk can be considered as a source of LAB strains with bacteriogenic potential (45, 46). The *E. mundtii* strain CRL 1656 isolated from cow's milk has been reported as bacteriocin-producing strain and showed a bacteriocigenic activity against the pathogen *Listeria monocytogenes* Scott A and *L. innocua* 7. This strain also able to produce a good amount of hydrogen peroxide, another inhibitory substance produced by LAB. Its use as a probiotic in cows has been recommended (46). Another strain, *E. mundtii* EM ML2/2, isolated from raw goat milk, produced a bacteriocin substance and showed an optimal activity at pH 6.3 (47).

The two other LAB strains, both isolated from meat sheep, with a bacteriostatic (248D) and bactericidal (120B) potential were identified as *Enterococcus hirae*. These results evince that different antimicrobial effects against Ma can be observed for different strains of same LAB specie. Other strains of *E. hirae* ST57ACC and DF105Mi have shown antimicrobial activity against *L. monocytogenes* by producing bacteriocins capable of resisting food processing (25, 45). A strain isolated from GM was also able to modulate the gut microbiota in dogs and did not present any virulence gene (43).

Regarding the evaluation of the commercial probiotic, the addition of L2 in GM (C3) showed a significantly higher bactericidal activity ($p < 0.001$) against Ma than that observed with strain 33B and 120B in GM (Tables 4, 6; C2-C5). This could also be related to the significant pH decrease observed throughout all experiments. Indeed, L2 significantly reduced the pH of the GM below five, when with Ma (Tables 4–6, C3), while strains 33B, 120B, or even 248D lowered the pH to values between 5.14 and 5.43 (Tables 4–6, C5). In previous studies, a similar inoculum was evaluated *in vitro* against Mb in bovine diluted semen and cervical mucus of cattle, and a significant reduction in the pH was also observed (28, 29). Consistent with these

studies, our results showed that L2 can also grow and acidify the extracellular medium in GM even when contaminated by Ma and could be a tool used as an antimicrobial strategy as it has been suggested (30). However, as mentioned previously, other possible influences such as competition for nutrients or the possible presence of bioactive peptides should not be ruled out as an antimicrobial mechanism of LAB (18).

These data regarding L2 could show a possible increase in the antimicrobial potential against Ma when several species of *Lactobacillus* spp. are used together as probiotics in GM. In this sense, the combination of various LAB strains is usually employed in commercial probiotics due to their synergy that increases their biological activity (48). The exact composition of this inoculum or one of similar composition had not been evaluated in previous studies (28, 29). In the present study, metagenomic analysis of the conditions with L2 evidenced for the first time, the real composition of this inoculum developed from a commercial probiotic for human use. The results showed that indeed, three species of *Lactobacillus* spp. are inoculated with our protocol (Figure 1). Our metagenomic study of the dynamics of the three species of *Lactobacillus* spp. of L2 showed that *L. brevis* increased its concentration to the detriment of *L. crispatus* in GM contaminated with Ma or not while *L. gasseri* had a steady RA over time. This provides a first approximation of the dynamics of these lactobacilli species in two different media and the possible role of *L. brevis* in the inhibition of Ma in GM. This specie has been isolated in raw milk of goats (49) and seems to have an antimicrobial effect against several pathogens such as *Bacillus cereus* (50), *Escherichia coli, S. aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (51). Nevertheless, it was reported that *L. brevis* was unable to acidify milk during a 20.5 h fermentation at 37°C (50) and could therefore not be responsible for the significantly lower pH observed at T15 in GM (C2-C3) and use different mechanisms to inhibit pathogens. *Lactobacillus gasseri* has also been isolated in caprine raw milk (52, 53) and seems to be a main component of the human vaginal flora, as well as *L. crispastus* (54), although the latest has never been isolated in milk to our knowledge.

Generally, this work suggests the antimicrobial potential of LAB against Ma under *in vitro* conditions, an important pathogen of the mammary gland of small ruminants. The necessity to explore possible applications of LAB, present in the microbiota of the mammary gland, as a control and prevention strategy against small ruminants' mastitis was previously suggested (24). Different studies have demonstrated the positive effect of LAB and its metabolites on the welfare of farm animals. It has been shown that the use of probiotics based on LAB reduces the occurrence of pathogens in large-scale farms (55). The results of the present *in vitro* study would suggest the need to inoculate, *in vivo* in caprine and ovine models, the strains identified in this study with an antimicrobial potential against Ma. In this sense, a preliminary study developed an intravaginal inoculation method in ewes, with doses inferior to L2 of the commercial probiotic used in this study, which showed the first signs of anti-inflammatory effects and had no prejudicial effects on the animals' health (27).

On the other hand, from an epidemiologic point of view, our results show that LAB with a negative effect against Ma can be naturally present in the mammary gland of ewes (248D, 120B) and goats (33B) from endemic regions of CA (Table 1). In all the three herds where the strains with antimicrobial potential were isolated, the use of antibiotics was anecdotic. The herds where 33B and 248D were isolated did not have any CA outbreaks, at least in the last decade, although they did manifest symptoms compatible with CA in the past. On the contrary, the ovine flock where 120B was isolated, had a clinical history of CA a year before this study took place. It is known that after a clinical outbreak of CA, the affected herds usually become chronically infected. This is normally attributed to an equilibrium created between the host and the pathogen, depending on the immune status of the herd. Moreover, it is accepted that the infection is not usually eliminated after the use of antibiotics and vaccines (2, 56, 57). Our results show the existence of LAB with antimicrobial potential against Ma in a CA chronically infected herd (strain 120B, Herd B, Table 1). Curiously, approximately one year after of this isolation, a new episode of decreased milk production was observed in this herd in animals where Ma was isolated again but no LAB was isolated. Therefore, the isolation of LAB never coincided with that of Ma and vice versa. In the herd that had a clinical outbreak of CA at the time of this study (Table 1, herd I), LAB were not isolated either. We suggest the hypothesis that this type of bacterial population (LAB) could contribute to the maintenance of the apparent asymptomatic status of a high number of animals in infected flocks. Furthermore, we need to consider that pathogenic species of *Mycoplasma* in ruminants such as the ones associated to CA (3, 58, 59), in asymptomatic animals, are usually found in anatomic locations such as articular liquid, lymph nodes, brain or external auditive canal, perpetuating the infection in the herds. We propose that with this strategy the pathogens not only try to avoid the immune system and the antimicrobial therapy (1, 2) but also the cohabitation with bacterial groups with antimicrobial potential such as the LAB. Indeed, these LAB populations can be found in the microbiota of the epithelium of the respiratory, mammary and reproductive tracts (23, 24, 60), which are anatomical locations that are colonized by mycoplasma associated with CA and linked to excretion route (34). In this sense, in a previous study involving *Salmonella* sp., the isolation of LAB was less important in dogs that were positive to this pathogen (21). Based on this hypothesis, the use of antibiotics could harm the natural barrier, that LAB with antimicrobial capacity represent, in locations such as the mammary gland of small ruminants. The results reported here could be the first indication of an undervalued interaction of LAB with other microbial agents, such as Ma, and suggests the need to carry out new studies on the bacterial ecology in CA infected animals.

In conclusion, this study marks the first description of the antimicrobial potential of LAB against Ma, hence a possible new alternative to the antibiotics used for the control of CA. To the authors' knowledge, the assessment of the antimicrobial potential of wild LAB against mycoplasmas of the hominis group has not previously been reported. In this sense, the inoculum L2, elaborated from a human commercial probiotic based on *Lactobacillus* spp., evinces itself as a strategy capable of achieving the complete inhibition of Ma *in vitro* in GM. The presence of *E. hirae* and *E. mundtii* is also confirmed in ovine and caprine milk with an *in vitro* bacteriostatic or bactericidal capacity against Ma in milk. The interaction between LAB and Ma reported here suggests a possible role of LAB in the dynamics of mycoplasmosis that should be studied. Our results suggest the necessity to design further *in vitro* studies to characterize other aspects of these LAB strains, such as other functional properties, bio-preservation and safety, as well as try to understand the inhibitory mechanisms, in order to corroborate their probiotic potential. In addition, *in vivo* studies would be needed to confirm its antimicrobial potential against mycoplasmas associated with CA and its innocuity on animals' health.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/; OQ538168, OQ538169, and OQ538170.

Author contributions

MT, EB, and ÁG-M designed the study and wrote the manuscript with input from all authors. MT, JG, RT-P, and ÁG-M collected the samples. MT and RT-P processed and analyzed the samples. MT, EB, JG, RT-P, and ÁG-M performed the laboratory experiments. MT recollected and prepared the data. AS analyzed the data. MT, EB, AS, JC, CF, and ÁG-M interpreted the data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Exploring the elevation dynamics of rumen bacterial communities in Barn feeding cattle from 900 to 3,600 meters by full-length 16S sequencing

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The diversity and abundance of rumen microorganisms serve as indicators not only of the host's digestive and metabolic capacity but also of its health status. The complex microbial communities in the rumen are influenced to varying degrees by environmental adaptability. In this study, we collected 24 rumen fluid samples from 24 healthy male cattle in three regions of Yunnan, China. Using 16S rRNA amplicon sequencing data analysis, we examined the variations in rumen microorganisms among cattle fed at altitudes of 900m, 1800m, and 3,600m. Altitude-related environmental factors did not surpass phylogeny as the main driving force behind the convergent evolution of yellow cattle rumen microbiome composition. However, they did have an impact on the alpha diversity of the rumen microbiome and the coevolution of the core microbiome. The change in altitude noticeably influenced the diversity and richness of the rumen microbiota, highlighting the environmental effect of altitude. As altitude increased, there was an observed increase in the abundance of *Firmicutes* and *Bacteroidetes*, while the abundance of ruminal *Proteobacteria* and *Kiritimatiellaeota* decreased. Importantly, at the genus level, the core genus exhibited distinct dynamic changes as altitude increased. Ruminants exhibit the ability to adapt their gut type in accordance with altitude, thereby optimizing energy utilization, especially in high-altitude settings. These discoveries offer valuable insights into the coevolution of host–microbe interactions during ruminant adaptation to various altitudinal environments.

KEYWORDS

rumen microbiology, cattle, microbiome, bacteria, altitude

Introduction

The gut of animals harbors a vast population of microbes, and a growing body of research indicates that the intestinal flora is extensive and vital for animal nutrition and health (1–4). Alterations in the composition of gut microbiota can influence host phenotypes associated with digestion, development, immunity, and behavior (5). Bioactive metabolites produced by the

intestinal flora influence host physiological processes, immune system regulation, and hormone secretion (6, 7). The composition, diversity, and function of the microbial community are closely associated with factors such as animal species, diet, environment, and other variables (8–10). The complex interaction of the host genome, nutrition, and living environment governs the composition and activity of the intestinal flora (11). The interactions shape the functional composition of intestinal flora species and contribute to the response to environmental stress. The impact of animal intestinal microecology on host physiology has long been a focal point of ecological research, particularly under changing environmental conditions. For example, despite the challenging conditions encountered at high altitudes, many animals thrive and develop specific physiological mechanisms. The intestinal flora may play a crucial role in adapting to the plateau environment (12). Certain studies suggest that gut microbes play a role in helping animals adapt to high altitudes. The rumen microbial genes of yaks and sheep at high altitude showed a significant enrichment in the volatile fatty acid production pathway, while the rumen microbial genes of cattle at low altitude displayed an enrichment in the methanogenesis pathway (13). Pikas that have adapted to the cold and low-oxygen high-altitude environment at high altitudes demonstrated higher intestinal microbial diversity, volatile fatty acid concentration, and cellulose degradation ability compared to the pikas residing in low-altitude areas (14). Research findings indicate that rhesus monkeys in high-altitude environments exhibit a higher abundance of Firmicutes to Bacteroidetes in their intestinal flora, along with an elevated presence of ruminococcaceae and Christensenellaceae. These factors potentially contribute to their adaptation to high altitudes (15).

Yellow cattle exhibit remarkable adaptability to various altitude environments, making them an ideal model for exploring the co-adaptation between extreme plateau environments and altitude gradients. As a result, they offer an opportunity to investigate the impact of varying altitudes on the composition and functionality of intestinal flora abundance. Currently, there is limited research on the interaction between rumen microflora and hosts in ruminants at different altitudes. In our previous study, we observed significant effects of altitude on the rumen microbes of yaks (16). The objective of this study is to investigate significant variations in the rumen microbiota of cattle residing at different altitudes, thereby enhancing our understanding of how the rumen microbiota influences host adaptation to distinct habitats. The findings will provide valuable reference information for research in microbial medicine conducted in high-altitude environments.

Materials

All animals involved in this experiment have received approval from the Animal Protection and Utilization Committee of Yunnan Agricultural University, China, and have adhered to the guidelines of the Laboratory Animal Ethics Committee. The collection of experimental animal sources and samples was conducted in accordance with these regulations. Group H ($n = 6$) was located in the pasture of Tiancheng Lun Zhu Agricultural Products Development Co., Ltd., in the north of Shangri-La County. The experimental site had an average altitude of 3,600 meters and belonged to a temperate monsoon climate. The maximum average daily temperature was 13°C, the minimum average daily temperature was 1°C, the annual precipitation was 600 mm, and the relative humidity was 65%. Group L (n = 6) was situated in Jiangcheng Xinfutai Agricultural Development Co., Ltd., located in the west of Jiangcheng County. The average altitude of the site was 900 meters, and it belonged to a subtropical mountain monsoon humid climate. The average annual temperature was 18.1°C. Group M (n = 12) was positioned in Jinjiang Green Beef Cattle Breeding Co., LTD, in the southern part of Anning City. The site had an altitude of 1800 meters and experienced a subtropical climate. The annual average temperature was 14.9°C, with extreme maximum and minimum temperatures of 31.5°C and −7.8°C, respectively. All three experimental groups were fed in barns with a diet consisting of whole silage maize and Milling Corn, as outlined in Table 1, which provides information about the dietary composition and nutritional levels. Table 2 presents the effects of different elevations on yellow cattle fattening. The feeding period lasted for 90 days, during which the animals' weights were measured on the first and last days before morning feeding. Two hours after the final morning feed, a catheter was inserted into the rumen, and rumen fluid samples were collected using a vacuum sampler. For each animal, 30 mL of rumen fluid was collected and divided into three parts, each placed in a 10 mL polypropylene tube and rapidly stored in liquid nitrogen. The samples were transported to the laboratory and stored in a refrigerator at −80°C.

DNA extraction and sequencing

The microbial community DNA was extracted using the EZNA Stool DNA Kit (Omega Bio-Tek, Norcross, Georgia, United States), following the manufacturer's instructions. The DNA was quantified using a Qubit Fluorometer and the Qubit dsDNA BR Assay kit (Invitrogen, USA), and the quality was assessed by running an aliquot on a 1% agarose gel. The variable regions V1–V9 of the bacterial 16S rRNA gene were amplified using degenerate PCR primers, 27F (5'-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5'-RGYTACCT TGTTACGACTT-3′) (17). Both the forward and reverse primers were tagged with Illumina adapters, pad, and linker sequences. PCR enrichment was carried out in a 50 μL reaction containing 30 ng of template, fusion PCR primer, and PCR master mix. The PCR cycling conditions were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 45 s, with a final extension

TABLE 1 Nutrient composition of whole corn silage (dry matter basis except for dry matter conten that is fresh basis).

Diet	Items	Nutrient ratio
Whole plant corn silage	Dry matter (%)	45.73
	Ash content (%)	7.40
	Crude protein (%)	15.83
	Crude fat (%)	3.16
	Acid Detergent Fiber (%)	33.21
	Neutral Detergent Fiber (%)	59.03
	crude fibre (%)	10.20
	Calcium (%)	1.14
	phosphorus (%)	0.27

TABLE 2 Effects of different elevations on yellow cattle fattening.

Ration level	Low altitude group (L)	Medium altitude group (M)	High altitude group (H)
DMI (kg/d)	6.80 ± 0.01	6.74 ± 0.14	5.75 ± 0.09
Initial Weight(kg)	229.08 ± 38.62	237.08 ± 44.53	163.71 ± 17.62
Fattening Period(d)	90	90	90
Final Weight(kg)	312.33 ± 44.99	318.08 ± 48.08	236.92 ± 15.04
Total weight gain during fattening(kg)	83.25 ± 13.45	81.00 ± 10.55	73.21 ± 12.68
ADG (kg/d)	0.93 ± 0.15	0.90 ± 0.12	0.81 ± 0.14

at 72°C for 10 min. The PCR products were purified using AmpureXP beads and eluted in Elution buffer. The libraries were assessed using the Agilent 2,100 bioanalyzer (Agilent, United States). The validated libraries were sequenced on the Illumina MiSeq platform (BGI, Shenzhen, China) using the standard Illumina pipelines, generating 2 × 300 bp paired-end reads.

Sequence analyses

The raw data were filtered to eliminate adapter contamination and low-quality readings, resulting in clean reads. The paired-end reads with overlaps were then merged to form tags. These tags were subsequently clustered into Operational Taxonomic Units (OTUs) at a 97% sequence similarity. Taxonomic ranks were assigned to representative sequences of the OTUs using the Ribosomal Database Project (RDP) Naive Bayesian Classifier v.2.2. Alpha diversity, beta diversity, and the identification of different species were analyzed based on the OTUs and taxonomic ranks. The clustering of tags into OTUs was performed using USEARCH (v7.0.1090) software. The taxonomic classification of the OTU representative sequences was done using the Ribosomal Database Project (RDP) Classifier v.2.2 trained on the Greengene_2013_5_99 database, with a cutoff confidence value of 0.5. The filtered tags were clustered into OTUs at 97% similarity. The number of OTUs per sample primarily represents the sample's diversity level. The OTUs of each group were listed, and Venn diagrams were created using the Venn Diagram software in R (v3.1.1) to summarize the common and specific OTU IDs.

Based on the abundance information of the OTUs, the relative abundance of each OTU in each sample was calculated. Principal Component Analysis (PCA) of the OTUs was performed using the relative abundance values with the ade4 package in R (v3.1.1). Good's coverage, alpha diversities (including Inverse Simpson and Shannon indices), richness (observed number of OTUs), and evenness (Shannon evenness) were calculated using Mothur V.1.31.2. Beta diversity analysis was conducted using QIIME (v1.80). Since there were differences in sequencing depth among the samples, normalization was introduced by randomly extracting sequences according to the minimum sequence number across all samples. The extracted sequences formed a new 'OTU table biom' file, and the beta diversity distance was calculated based on this file. Statistical results, including beta diversity differences between groups, species abundance histograms, and histograms comparing differences in key species, were plotted using R (v3.4.1). Bacterial community typing was conducted using R (v3.4.1). KEGG function prediction was performed using R (v3.2.1) and the software PICRUSt2 v2.3.0-b. The LEfSe software was utilized for differential species analysis.

Results

Analysis of rumen microbial diversity

A total of 1,644 OTUs were identified in the three experimental groups: high altitude, middle altitude, and low altitude. The high altitude group had 1,355 OTUs, the middle altitude group had 1,374 OTUs, and the low altitude group had 1,144 OTUs. As shown in Figure 1A, a total of 889 OTUs were present in the three experimental groups, with 177 OTUs unique to the high-altitude group, 101 OTUs unique to the medium-altitude group, and 26 OTUs unique to the low-altitude group.

Alpha diversity was evaluated using parameters such as the Observed species index, Chao index, ACE index, Shannon index, Simpson index, and Good-coverage index based on abundance (Figure 1B). The Observed species index, Chao index, ACE index, and Shannon index showed an increasing trend in the low, middle, and high altitude groups, indicating that the diversity and richness of rumen microbiota in the high altitude group were the highest ($p < 0.05$). Moreover, the Simpson index, which reflects the species diversity of the communities, showed that the diversity of rumen microorganisms in the high-altitude group was higher than that in the medium-low altitude group.

We detected 19 phyla (Supplementary Figure S1A) in the samples from the three elevation regions, which accounted for more than 0.1% of the community abundance at the phylum level. The dominant phyla were Bacteroidetes, Firmicutes, Proteobacteria, and Kiritimatiellaeota. The relative abundance of Firmicutes and Bacteroidetes was 21.55, 23.41, and 31.03% at low, middle, and high altitudes, respectively, while Kiritimatiellaeota had relative abundances of 28.62, 34.64, and 38.52% at the same altitudes. This trend indicated an obvious increase in relative abundance with increasing altitude (Figure 2). The ratio of Firmicutes to Bacteroidetes was 0.75, 0.67, and 0.80 in yellow cattle at low, middle, and high altitudes, respectively.

The abundance of Proteobacteria and Kiritimatiellaeota in the rumen exhibited a decrease as altitude increased. Additionally, the relative abundance of Firmicutes, Lentisphaerae, and Fibrobacteres showed significant differences among yellow cattle rumen microorganisms at low, medium, and high altitudes. In the 24 samples analyzed (Supplementary Figure S1B), we identified 33 genera, with *Prevotella* and *Kiritimatiella* being the most abundant across all three elevation levels in the rumen of yellow cattle. Notably, there were distinct and dynamic changes observed at the genus level in ruminal bacteria as altitude increased.

Elevation environment and differential microbes

The prokaryotic community composition in the rumen exhibits significant variations at both the phylum and genus levels. To

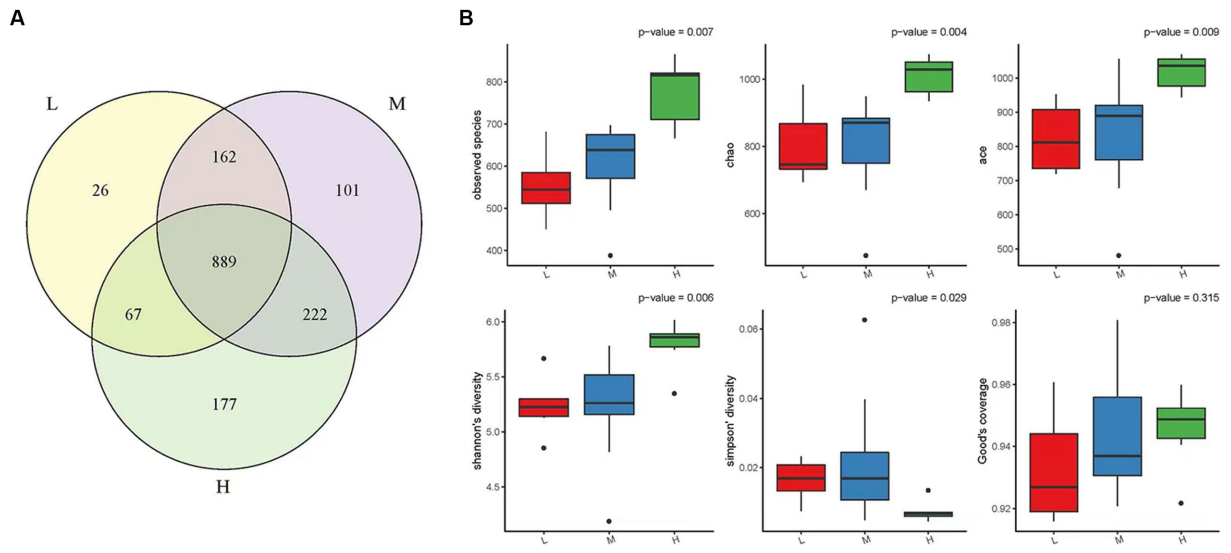

FIGURE 1
(A) OTU Venn diagram. In the Figureure, different color graphs represent different samples or different groups, and the number of overlapping parts is the number of OTUs shared between two samples or two groups. Similarly, the number of multiple overlapping parts refers to the number of OTUs shared among multiple samples or groups. Low altitude group (L), medium altitude group (M), high altitude group (H). **(B)** Alpha diversity box chart. The Observed Species index, Chao index, ACE index, Shannon index, Simpson index and Good-coverage index are included. The larger the first four indices, the smaller the fifth index, the more abundant the species in the sample. Low altitude group (L), medium altitude group (M), high altitude group (H).

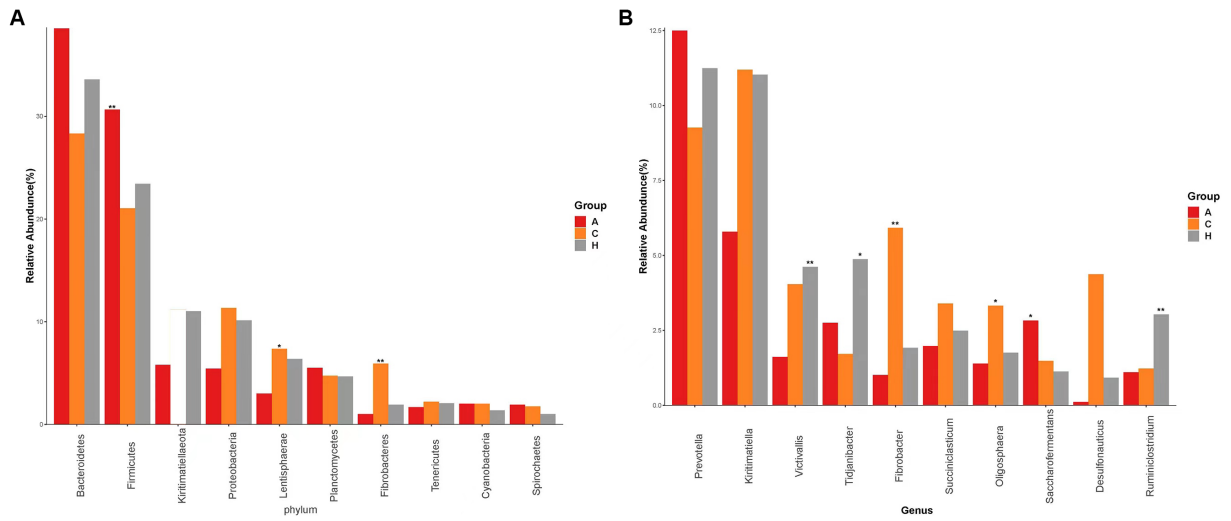

FIGURE 2
Species with the top 10 abundence, showing the average relative abundence of each group and the significance of the difference test (marked with an "*" at the top of the bar chart if any, not marked if not). **(A)** Comparison of dominant phyla in Low altitude group, Medium altitude group, High altitude group. **(B)** Comparison of dominant genera in the Low altitude group, Medium altitude group, High altitude group. Low altitude group (L), medium altitude group (M), high altitude group (H).

investigate the differential microbial communities among the low, middle, and high altitude groups, we utilized linear discriminant effect sizes (LEfSe) analysis, including LDA (linear discriminant analysis) (Figure 3A). The LEfSe results showed that microbial groups with significant effects were displayed in different colors in the low, middle, and high altitude groups. Among these groups, the high altitude group had the largest number of different microorganisms (Supplementary Figure S2). The signature gut microbiota in the low-altitude group included Fibrobacteria, Fibrobacteraceae, and Lentisphaerae. Victivallaceae was predominant in the medium-altitude group, while Bacteroidetes and Clostridial were prominent in the high-altitude group. Considering the reports suggesting that intestinal type can reflect functional differences, we investigated whether the rumen bacterial community of yellow cattle could be categorized into functional groups based on altitude variations. Principal component analysis (PCA) revealed distinct intestinal types

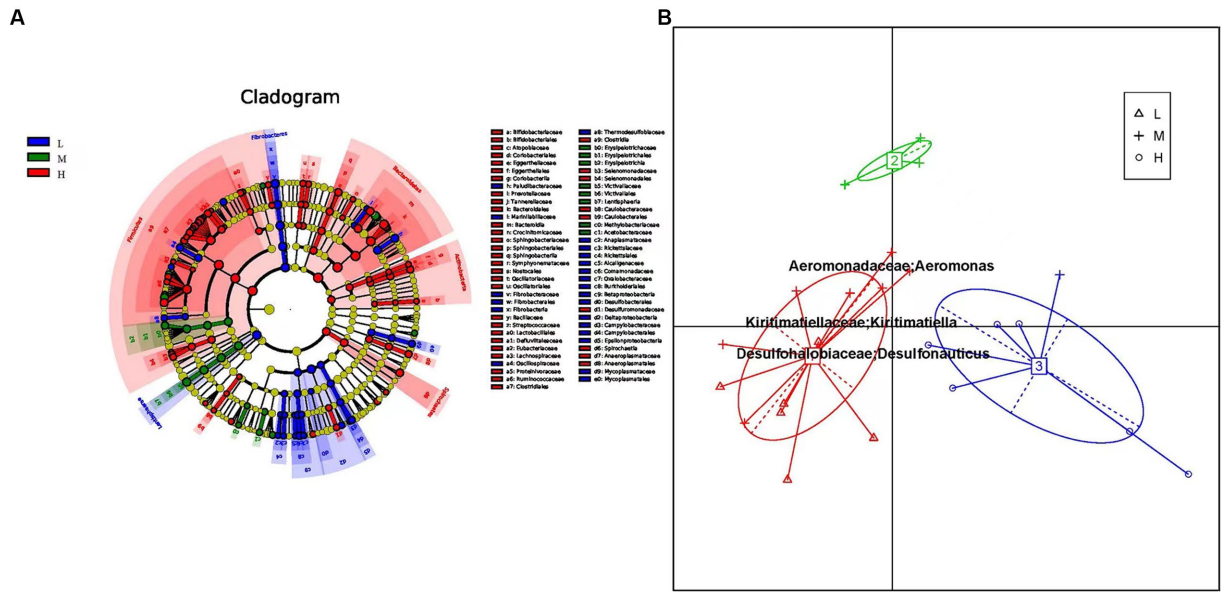

FIGURE 3
(A) Cluster plots were analyzed by LEfSe. Different colors represent different groups, nodes of different colors represent the microbiota that play an important role in the group represented by the color, a color circle represents a biomarker, and the legend in the upper right corner is the biomarker name. Yellow nodes indicate microbial taxa that did not play an important role in the different groupings. From the inside to the outside, each circle is divided into phylum, class, order, family, and genus level species. **(B)** Enterotypes analysis. The abscissa represents principal component one, and the ordinate represents principal component two, which are the two principal components with the largest variance contribution rate.

formed by the samples through Bray-Curtis differential analysis. Each cluster was characterized by changes in the abundance of its representative genus, Enterotype 1 exhibited Kiritimatiella and Desulfonauticus, while Enterotype 2 showed a high abundance of Aeromonas (Figure 3B).

Predicted function and metabolism of rumen microbiota

The predictions of bacterial community KEGG function abundance were obtained using PICRUST2. In the low, middle, and High altitude groups, the relative abundances of Metabolism and Genetic Information Processing were 79.72 and 14.44%, respectively (Figure 4A). A total of 29 biochemical pathways were identified among the metabolic functions. Functions related to Metabolism of cofactors and vitamins, Carbohydrate metabolism, and Amino acid metabolism were enriched in all samples (Figure 4B). Additionally, the microflora of the low, middle, and high altitude groups exhibited other functional roles, such as cellular processes, organismal systems, environmental information processing, human diseases, and genetic information processing.

Discussion

Animal gut microbes are influenced by various factors, including diet, genetics, age, environment conditions such as altitude and geographical location (18–21). This study focuses on exploring the relationship between rumen bacterial composition and function in Yellow cattle with respect to altitude. Gut microbes play an important role in host adaptation to different diverse environments (22, 23), providing essential nutrients and maintaining intestinal homeostasis (24, 25). Previous studies have demonstrated that different elevations have specific effects on the composition and fermentation function of rumen microbiomes in grazing yaks (26). Furthermore, it has been observed that the altitude environment drives convergent evolution of α diversity and indicator microbiota in animal gut microbiota (27). The intestinal microbiota of hosts exhibits distinct characteristics according to different altitude habitats (28). The shared features of intestinal microbiota at various elevations suggest a co-evolution between mammalian gut microbiota and their hosts (29). Numerous studies have demonstrated that alterations in altitude can influence the changes in intestinal microecology, subsequently impacting the structure and function of mammalian intestinal flora (30). Intestinal population diversity is profoundly influenced by altitude, with notable distinctions observed between high altitude and low altitude populations. The intestinal microbial communities in yaks at different altitudes were dominated by Firmicutes (63.42%) and Bacteroidetes (47.4%) at the phylum level (16). Due to cold stress, ruminants at high altitudes may experience an increased reliance on carbohydrates, necessitating Firmicutes and Bacteroidetes to supply additional energy for maintenance purposes.

The interaction between intestinal flora and host not only regulates metabolism, but also serves as a crucial bridge connecting the environment and host, thus helping the host better adapt to different environments (17). The diversity analysis revealed an increasing trend in both the diversity and uniformity of rumen bacteria among cattle in low, middle and high altitude areas. In this study, Yellow cattle from all altitude regions were fed the same diet to maintain uniformity, highlighting altitude as the

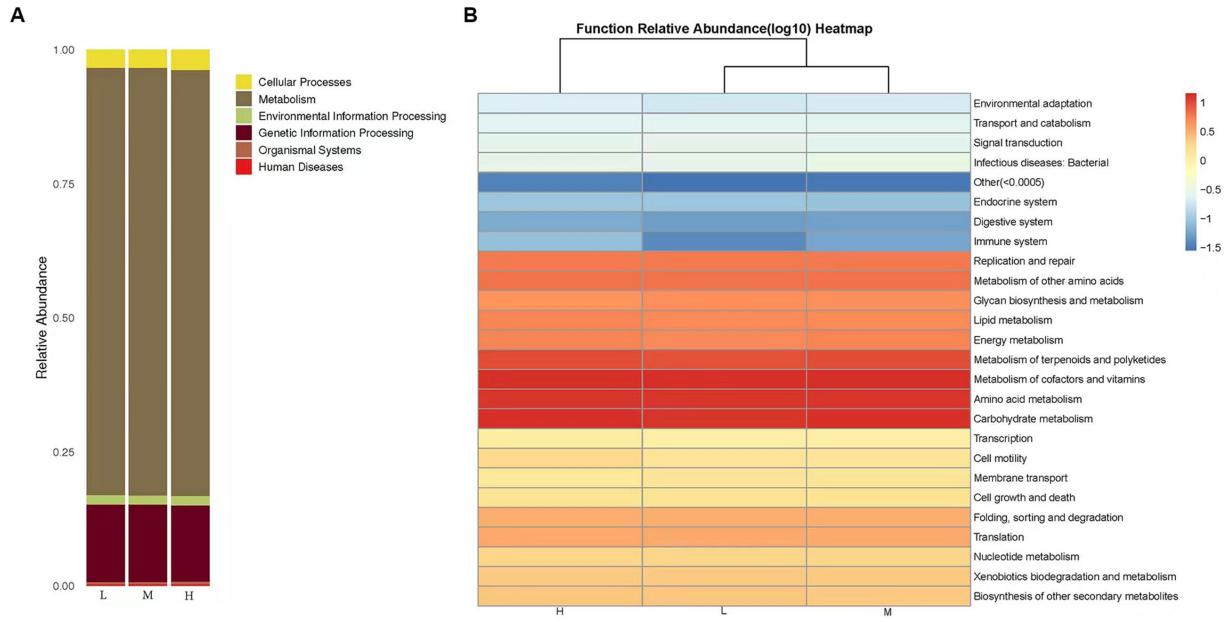

FIGURE 4
(A) KEGG classification bar chart. The predicted microbiota function based on the KEGG database, the horizontal axis represents the sample, and the vertical axis represents the relative abundance of the predicted function. **(B)** Heat map of functional predicted abundance. Longitudinal clustering indicates the similarity of functional prediction among different samples. The closer the distance, the shorter the branch length, indicating that the functional prediction and abundance of samples are more similar. Horizontal clustering indicates that the abundance of this function is similar in each sample.

primary factor influencing the changes in rumen microbial diversity. Previous studies have demonstrated higher rumen bacterial community diversity and rumen fluid volatile fatty acid content (VFA) in yaks at an altitude of 4,700 m above sea level on the Qinghai-Tibet Plateau compared to those at middle and low altitudes (31). Through sample clustering, it was observed that the rumen bacteria of Yellow cattle at low, middle, and high altitudes did not primarily group together in the evolutionary branch, but rather individuals within the same altitude exhibited clustering. In terms of the number of endemic microorganisms, the number of endemic rumen bacteria at high altitude was significantly higher than that at middle and low altitudes, but most of the core microorganisms at the three altitudes were common. The co-evolution of the host-gut bacterial system has formed a common core microbe under the influence of different elevations (32).

The meadow at different elevations exhibit variations species richness and the forage found within them possesses varying nutritional value (33). As a result, the high-altitude group displayed significantly greater bacterial diversity compared to the low-altitude group. It is generally observed that higher gut bacterial diversity and richness are associated with a healthy and stable host gut microbiome (34). In contrast to the low altitude group, the high altitude group exhibited a higher abundance of Bacteroidetes and Firmicutes in the rumen. These bacteria play a crucial role in the decomposition of fibers and cellulose, providing the necessary energy for the host (35). Furthermore, the high altitude group displayed a noticeable upward trend in the Firmicutes/Bacteroidetes ratio compared to the low and middle altitude groups. The elevated ratio of Firmicutes to Bacteroidetes in the rumen of the high altitude group indicates a greater propensity for fat deposition (36, 37). Studies have revealed a significant difference in the ratio of Firmicutes to Bacteroidetes in the gastrointestinal microbiome between high-altitude and low-altitude ruminants. The higher ratio observed in high-altitude ruminants has been shown to impact energy deposition (38). Altitude affects the energy metabolism of the gut microbiome and the ability to decompose substances such as fiber and cellulose (39). Kiritimatiellaeota is involved in the biosynthetic pathway of arginine and fatty acids, thereby utilizing nitrogen in food and producing energy (23). In this study, the abundance of rumen Kiritimatiellaeota in the middle-high altitude group was found to be significantly lower than that in the middle-low altitude group. Kiritimatiellaeota plays a particularly crucial role in the rumen of herbivores (40).

Compared to the high altitude group, the middle and low altitude groups exhibited greater activity in the biosynthetic pathways of arginine and fatty acid. In response to altitude fluctuations, the gut microbiome can adapt its metabolic rate and enhance the extraction of energy from complex carbohydrates, thereby promoting co-evolution between the host and the microbes. *Prevotella*, the genus with the highest abundance in the rumen across all altitude groups, signifies optimal digestive dynamics and contributes to intestinal homeostasis. Hence, a higher diversity of *Prevotella* and other fiber-degrading microorganisms enhances the microbiota's ability to ferment, promoting gut health (41). Research has indicated that a high *Prevotella-Bacteroides* ratio can impact fiber digestion and glucose metabolism (42). The ratio of *Prevotella-Bacteroides* in the rumen

of the middle-high altitude group was significantly higher than that of the low-altitude group. This long-established host-*Prevotella* symbiosis, developed through hundreds of thousands of years of coevolution, can result in compromised host-microbial interactions, consequently impacting host health.

The relative abundances of *Tannerella*, *Prevotella* and *Eubacterium* increased with increasing altitude. The host's physiological responses to altitude, such as changes in immune function and metabolism, can impact the microbial community composition. In addition, Firmicutes/Bacteroidetes in the high altitude group showed an obvious upward trend compared with the low and middle altitude group. The ratio of Firmicutes to Bacteroidetes in the rumen of the high altitude group was higher, indicating better fat depositiony create an environment where *Tannerella*, *Prevotella*, and *Eubacterium* thrive and establish higher relative abundances compared to other microbial groups. The increase in the relative abundances of *Tannerella*, *Prevotella*, and *Eubacterium* with higher altitude can be attributed to a combination of environmental factors, host physiological adaptations. The decrease in the relative abundance of *Fibrobacter* and *Kiritimatiella* with increasing altitude could be attributed to changes in environmental conditions. On the other hand, the significantly higher relative abundance of *Butyrivibrio* in the high altitude group suggests its ability to adapt and thrive in the unique conditions found at higher altitudes, potentially influenced by both environmental factors.

This study provides valuable insights into intestinal flora and its functionality. We elucidate the rumen bacteria composition and functional genome information in farmed cattle across different altitudes. Among these findings, Kiritimatiellaceae intestinal types are predominantly observed in the low-to-mid-altitude group, while Desulfonauticus intestinal types are more concentrated in the high-altitude group, likely due to their adaptation to cold environments. Previous research has demonstrated that the proportion of dietary carbohydrate content in baboons directly influences the transformation of the host intestinal pattern. Therefore, changes in altitude-specific dietary protein and carbohydrate content may offer an intriguing explanation for the dynamics of enterotypes and assist in identifying the enterotype of high-altitude ruminants (43). In this study, the most notable evidence of elevation-induced changes in intestinal types was observed in type 2 and type 3 at middle and high altitudes, while type 1 remained stable at low altitudes. This finding suggests that the long-term co-evolution between the host and the environment contributes to distinct dynamics of intestinal types, playing a vital role in ruminant formation and adaptation to high-altitude extreme environments. The functional attributes of the intestinal microbiome govern the interactions between the host and the microbiome, ultimately shaping their mutual relationship (44). The findings from PICRUSt2 analysis revealed distinct variations in the rumen microflora of Yellow cattle across the three altitude regions, with metabolism being the most prominent and active function. Specifically, carbohydrate metabolism and amino acid metabolism were predominant. Interestingly, our study also uncovered a striking similarity in the functional genetic composition of rumen microbes among cattle at the three elevations. These results imply that rumen bacteria in cattle exhibit a heightened sensitivity to environmental adaptability compared to gut bacteria.

Conclusion

Altitude environmental factors do not supersede phylogeny in driving the convergent evolution of the yellow cattle rumen microbiome composition. However, they do exert an influence on rumen microbiome alpha diversity and the coevolution of the core microbiome. Notably, certain key genera, including *Tannerella*, *Ruminobacter*, and *Prevotella*, demonstrate associations with the altitude environment. Our findings suggest that high-altitude regions provide a more favorable environment for rumen bacterial fermentation compared to low-altitude or medium-altitude areas. Furthermore, there may exist convergent evolution between the core microbiome and the host. These results indicate that rumen microorganisms in Yellow cattle from high-altitude areas have adapted to extreme environments, enabling them to maximize feed utilization efficiency.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

All animals used in this experiment were approved by the animal protection and utilization committee of Yunnan Agricultural University, China (protocol # 2018–009), and there was compliance with the guidelines of the Laboratory Animal Ethics Committee in experimental animal handling.

Author contributions

DW and SY made substantial contributions to the conception or design of the experiments. SY and ZY performed the experiments. RW and SH analyzed the data. DW and ZY wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1169573/full#supplementary-material

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Effect of dry cow therapy on antimicrobial resistance of mastitis pathogens post-calving

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The aim of this study was to evaluate the effect of dry cow therapy (DCT) on the antimicrobial resistance (AMR) profile of mastitis pathogens post-calving. A repository of isolates based on a DCT trial was utilized for the current study. A stratified random survey sample of cows from the trial were identified within the strata of season, herd, and trial treatment resulting in 382 cows. All isolates from the 382 cows were selected for the current study, which identified 566 isolates from milk samples collected at dry off (S1), post-calving (S2), and at the first clinical mastitis event up to 150 days in milk (S3). The AMR profiles were determined using broth microdilution method. Less than 10% of the coagulase-negative *Staphylococcus* species (CNS) isolates ($n = 421$) were resistant to tetracycline, ceftiofur, penicillin/novobiocin or erythromycin, while higher proportions of resistance to sulfadimethoxine (72%) and penicillin (28%) were observed. All *Staphylococcus aureus* (*S. aureus)* isolates ($n = 4$) were susceptible to all tested AMD except sulfadimethoxine, to which all isolates were resistant. Similarly, all *Streptococcus* spp. ($n = 37$) were susceptible to penicillin, penicillin/novobiocin, and ampicillin while resistant to tetracycline (17%). All coliforms ($n = 21$) were susceptible to ceftiofur, but resistance was recorded for sulfadimethoxine (70%), cephalothin (56%), and tetracycline (43%). The increased resistance percent from S1 to S2 was observed in CNS isolates from AMD-treated cows, with the highest increase recorded for penicillin (12.2%). Parametric survival interval regression models were used to explore the association between antimicrobial drug (AMD) therapy at dry off and the AMR phenotype post-calving. The accelerated failure-time metric was adopted to minimum inhibitory concentration measurements to permit interpretation of model exponentiated coefficients. Models for cows with CNS isolated at both S1 and S2 showed increased resistance against cephalothin, oxacillin, and ceftiofur in cows that received DCT from the same drug class, or a class with a shared resistance mechanism. In contrast, resistance of CNS isolates to tetracycline were associated with any AMD therapy at dry off. Resistance of CNS isolates to Penicillin decreased in CNS isolates in cows that received any AMD therapy at dry off compared to those that didn't. The study provided evidence that dry-cow IMM AMD was associated with AMR post-calving.

KEYWORDS

dry cow therapy, antimicrobial resistance, mastitis pathogens, coagulase negative *Staphylococcus* (CNS), *Streptococcus*, coliforms

1. Introduction

Mastitis is the most economically important disease of dairy cows and a major indication for antimicrobial drug (AMD) use on dairies (1). A recent USDA survey showed that clinical mastitis was detected in approximately one-fourth (24.8%) of all cows at some point in 2013, and cases of clinical mastitis were reported in almost all US dairy operations (99.7%) (2). The same report showed that intramammary antimicrobials were routinely administered to the majority of US dairy cows (89.9%) at dry off (2).

Antimicrobial therapy is a key component of mastitis control programs, commonly administered as an intramammary antimicrobial infusion (IMM) to treat clinical mastitis during the lactation (3), or administered at dry-off to treat existing subclinical infections and prevent new infections during the dry period and early post-partum period (4). At dry-off, intramammary antimicrobials are either administered to all cows (blanket dry cow therapy-BDCT) or selectively to cows at high risk for mastitis during the dry period and early post-partum period (selective dry cow therapy-SDCT). The latter approach is considered a judicious AMD use practice since AMD administration is limited to cows with elevated risk for mastitis that would more likely benefit from such treatment, such as cows with a history of clinical mastitis during the current lactation and cows with high milk somatic cell counts (SCC), which is an indication of subclinical intramammary infection (5).

In the US, a retrospective analysis of 8,905 bacterial isolates obtained from milk samples submitted to the Wisconsin Veterinary Diagnostic Laboratory between 1994 and 2001 showed no specific trend of resistance across drugs over time. For instance, the percentage of *Staphylococcus aureus* (*S. aureus)* isolates resistant to penicillin decreased from 49 to 30%, while percentage of Coagulase negative staphylococci (CNS) isolates resistant to pirlimycin increased from 6 to 19% over the study period which may be due to changes in underlying populations (6). In Canada, a study on resistance profiles of mastitis pathogens on Canadian dairy farms estimated low levels of resistance ranging from 0% (cephalothin and oxacillin) to 8.8% (penicillin) in *S. aureus* isolates, while the estimates for AMR in *Escherichia coli* (*E. coli)* ranged from 0% (ceftriaxone and ciprofloxacin) to 14.8% (tetracycline) (7). Similarly, a study conducted on 934 bacterial isolates from nine European countries during 2009–2012 showed varying levels of resistance to commonly used AMD with 1% resistance against ceftiofur (*S. aureus* and *E. coli*), 14.5, 5.2, and 36.7% resistance against tetracycline for *E. coli*, *S. aureus,* and *Streptococcus uberis*, respectively, and 25.0% resistance against Penicillin G in *S. aureus* (8).

Most of the previously mentioned studies utilized a cross-sectional study design with no specific information on the AMD exposures of the study cows (7–10). The current study objectives were (1) To utilize a longitudinal study design to characterize the changes in the AMR profiles of bacterial isolates from milk samples collected at dry off, post-calving, and the first mastitis event within 150 days in milk (DIM). (2) To assess the effect of dry cow therapy on antimicrobial resistance of mastitis pathogens in the subsequent lactation. Monitoring the AMD exposure and AMR profile of mastitis pathogens is vital in guiding management strategies to reduce AMD use and minimize the AMD resistance while protecting food safety, animal, and public health.

2. Materials and methods

2.1. Study design and sampling procedures

Bacterial isolates utilized in this longitudinal study were selected from a repository generated during a randomized blocked field trial conducted on eight California dairies between December 2016 to April 2018 (11). The original trial was approved by the University of California Davis Institutional Animal Care and Use Committee (protocol number 19761). A total of 1,106 cows were enrolled at dry-off on the eight dairies in two seasonal cohorts, fall/winter and spring/summer, and followed to 150 DIM. The study herds were distributed across Northern San Joaquin Valley (NSJV) and Greater Southern California (GSCA) (12). The original trial was conducted to estimate the effect of different dry cow treatments: (1) IMM antimicrobial infusion (AB); (2) Internal Teat Sealant (ITS); (3) Both AB and ITS (AB+ITS); and (4) no treatment (None) on health and production outcomes during the next lactation. The outcome variables evaluated for each treatment group included udder health, milk production and culling during the subsequent lactation. A stratified random survey sample was used to select 382 cows from the trial's 1,106 cows with proportional allocation across the strata season, herd, and treatment. A total of 566 bacterial isolates from milk samples of the 382 cows were utilized for the current study had comparable season, herd, and treatment distribution to the entire repository (Figure 1). Among the selected cows, 192 received IMM AMD infusion (AB or AB+ITS treatment groups) while 190 did not receive IMM AMD infusion and served as controls (ITS or none groups). Sampling stage represented the three timepoints when the milk samples were collected: at dry off and before treatment (S1), post-calving (S2), and at first mastitis event within the first 150 DIM (S3). Intramammary antimicrobial drug infusions used in the study were FDA-approved, commercially available products which included cloxacillin benzathine (Dryclox®, Boehringer Ingelheim) (45 cows), ceftiofur hydrochloride (Spectramast DC®, Zoetis) (16 cows), cephapirin benzathine (ToMORROW®, Boehringer Ingelheim) (85 cows) and a proprietary combination of procaine penicillin G and dihydrostreptomycin (Quartermaster®, WG Critical Care, LLC) (46 cows).

2.2. Bacterial culture and identification

Bacterial culture and identification were performed following standard protocols used by the National Mastitis Council at the Milk Quality Lab (MQL) at the UC Davis Veterinary Medicine Teaching and Research in Tulare, California (13). Briefly, milk samples were plated on bovine blood agar using calibrated sterile loops and incubated for 24 to 48 h at 37°C. Colony types were identified by colony morphology, hemolysis properties, Gram stain, and biochemical tests. *Staphylococcus aureus* was confirmed by a positive coagulase test; all coagulase-negative *Staphylococcus*

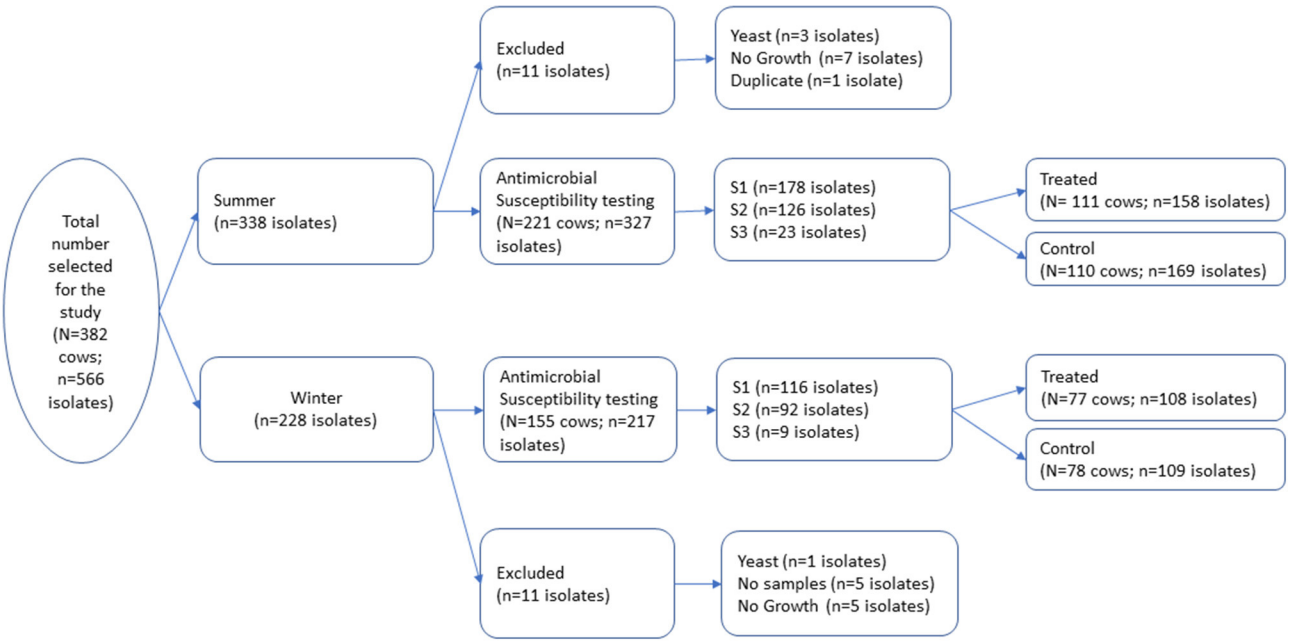

FIGURE 1
A total of 566 milk pathogen isolates representing 382 cows were selected from a repository of isolates generated from a block randomized field trial for dry cow therapy. Isolates were collected at dry off (S1), post-calving (S2), and at first clinical mastitis prior to 150 days in milk (S3).

species (CNS) isolates were reported as *Staphylococcus* spp. *Streptococcus* spp. were identified by a negative catalase test and *S. agalactiae* was identified by a positive CAMP test and a negative Esculin test. Gram-negative, KOH-positive bacteria were reported as coliforms. All isolates, except *Staphylococcus* spp., were identified to species level by partial sequencing and analysis of 16S RNA gene using 27f and 1492r primers pair as previously described (14).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility of the selected isolates was determined by estimating the minimum inhibitory concentration (MIC) using a commercial antimicrobial susceptibility test (AST) plate specific for mastitis pathogens (CMV1AMAF®; Sensititre®, Thermofisher) and following the manufacturer's procedure. Briefly, 1–5 fresh overnight (24 h) colonies of the bacterial isolate on blood agar (BA) media were resuspended in 5 ml of demineralized water [or 5 ml Mueller-Hinton broth (MHB) for *Streptococcus* spp.] and the concentration adjusted to 0.5 McFarland standard. Next, 10 µl (30 µl for *Streptococcus* spp.) of the bacterial solution was added to 11 ml MHB (or MHB with hemolyzed horse blood for *Streptococcus* spp.) and mixed by repeated inversion of the tube. Fifty microliters of inoculated MHB media were added into each well of the 96-well CMV1AMAF® plate and incubated at 37°C for 18–24 h. The purity and bacterial count in the inoculated MHB broth was checked by taking 1 µl inoculum sample from a positive control well in the AST, streaked on BA, and incubated at 37°C for 18–24 h. The AST plates that had contamination or no growth on corresponding BA were not read, and the test was repeated. The MIC values were read using Sensititre™ Vizion™ Digital MIC Viewing System. The MIC values were recorded as the lowest concentration of antimicrobial drug that inhibited the growth of bacteria. The CMV1AMAF® AST plate contained 10 antimicrobial drugs: ampicillin, penicillin, erythromycin, oxacillin, pirlimycin, penicillin/novobiocin, tetracycline, cephalothin, ceftiofur, and sulfadimethoxine. Susceptibility of the tested isolates against different antimicrobial agents was determined based on CLSI breakpoints (CLSI 2019; VET08, 4th ed). For AMD that did not have established clinical breakpoints the distribution of the MIC values were reported.

2.4. Data analyses

The distribution of bacterial isolates by species and seasonal cohort, and susceptibility of the different species against AMD tested were summarized as percentages. The distribution (number) of isolates by MIC interval for each of the drugs in the mastitis AST plate (CMV1AMAF) were summarized by season. Statistical analyses were performed using Stata software (Stata Corp. 2017. Stata Statistical Software: Release 15. College Station, TX: Stata Corp LLC).

2.5. Modeling the effect of treatment on MIC values

Models were limited to cows with the same bacterial species isolated at S1 and S2 ($n = 90$ cows). Each model explored the association between AMD therapy at dry off and the AMR phenotype for the drugs available on the CMV1AMAF AST plate.

Isolate resistance to a specific drug was measured as a range of MIC values with the lower or upper limits censored (left or right censored, respectively). The original trial treatment groups were explored as an explanatory variable in the model, specifically, AB, ITS, AB+ITS vs. None. Alternative specifications of the treatment variable were explored including a dichotomy comparing AMD therapies (AB or AB+ITS) vs. no AMD therapies at dry off (ITS or None); and based on the type of AMD administered at dry off comparing exposure to, vs. lack of exposure to the dry cow AMD, namely, penicillin (penicillin–dihydrostreptomycin), cephalosporins (ceftiofur hydrochloride or cephapirin benzathine), or cloxacillin benzathine (a semisynthetic beta-lactamase resistant penicillin). Other explanatory variables explored included herd, parity, region, seasonal cohort, breed(s), most recent and highest SCC based on the 6 monthly test records prior to trial enrollment (at dry off), and history of mastitis in the enrollment lactation. In addition, specific observations at enrollment were explored including; California Mastitis Test (CMT) score (negative, trace, 1, 2 or 3) (15); teat end score (1–4; 1 = normal and 4 = very rough cracked teat end with ring); and udder hygiene score (1–4; 1 = clean and 4 = dirty) (16). Finally, the antimicrobial resistance phenotype at S1, and the length of the period between S1 and S2 sampling dates (days) were explored as model covariates.

Interval regression models assume censoring occurs from a normally distributed outcome which may not be true for MIC results. Alternatively, parametric survival interval regression models can be used to model the association between AMD therapy at dry off and the AMR phenotype post-calving. For each drug, AMR was modeled using the exponential, Weibull, Gompertz, lognormal, loglogistic, or generalized gamma distributions. The best fitting parametric distribution was selected based on the lowest Akaike Information Criterion (AIC) estimate for the respective parametric distribution intercept only model. In addition, in the case of the Weibull distribution, its shape parameter (p) and its statistical significance test (H_0: $p = 1$) was used to confirm whether an exponential or a Weibull distribution was better fitting. Specifically, Weibull distribution was selected over the exponential if the null hypothesis was rejected since the Weibull distribution is reduced to exponential when $p = 1$ (17).

For all models, the accelerated failure-time (AFT) parametrization (instead of hazard) was implemented. The susceptibility of an isolate, measured in MIC was modeled for each drug with robust estimates for standard errors to account for clustering of observations by dairy. To permit interpretation of the model exponentiated coefficients, we introduce the novel nomenclature of an MIC ratio. Identical to AFT model exponentiated coefficients presented as time ratios, the MIC ratio is the quotient resulting from dividing the MIC estimate for a specific covariate profile that represents the exposed (numerator), by that of the unexposed (denominator). As such, an MIC ratio varies from 0 to infinity with 1 indicating no difference between the exposed and unexposed, < 1 indicating that the exposure is protective, or > 1 indicating the exposure is a risk factor. To aid in interpretation, the model predicted MIC were estimated for isolates from cows by dry off treatment status.

Once the best parametric distribution was identified for resistance against each of the study panel's drugs, additional univariate models were specified before the final models were determined using a manual forward building approach. The best fitting model had the lowest AIC value (18) and produced estimates that were within the maximum possible MIC drug concentration (<1,000,000 μg/ml). Confounding was assessed using the method of change in estimates and biologically plausible interactions determined using statistical significance testing (19).

3. Results

3.1. Bacterial isolates

A total of 566 isolates were initially selected for the antimicrobial susceptibility testing, but 22 isolates were excluded due to contamination, no growth, missing sample, or duplicate samples as shown in Figure 1. The remaining 544 isolates that were tested for antimicrobial susceptibility are summarized in Table 1. The most common isolates were CNS ($n = 421$), *Streptococcus* spp. ($n = 37$) and *E. coli* ($n = 19$). No *Streptococcus agalactiae* or *Mycoplasma* spp. were isolated from any of the samples. The 16S RNA gene sequences of the isolates were submitted to the GenBank (Accession: OR142768-OR142978).

3.2. Antimicrobial susceptibility

Table 2 summarizes the percent susceptibility of the study isolates to the 10 AMD tested. The four *S. aureus* isolates were susceptible to all AMD tested except sulfadimethoxine, to which all isolates were resistant. More than 90% of the CNS isolates, the most common of all isolates, were susceptible to tetracycline, ceftiofur, penicillin/novobiocin, pirlimycin, and erythromycin. The lowest susceptibility estimate for CNS isolates was for sulfadimethoxine (28%) followed by susceptibility to penicillin (72%). All the *Streptococcus* spp. isolates were susceptible to ampicillin, penicillin and penicillin/novobiocin with more than 90% of the isolates susceptible to erythromycin, pirlimycin, and ceftiofur. In contrast, 17% of *Streptococcus* spp. isolates were resistant to tetracycline. All coliforms (*E. coli* and *Klebsiella* spp.) isolates were susceptible to ceftiofur, while 43% were resistant to tetracycline and 56% were resistant to cephalothin. The lowest susceptibility for coliforms was recorded against sulfadimethoxine (30%).

The distribution of MIC values for the CNS isolates, stratified by season, are summarized in Tables 3, 4. The distribution of MIC values for the CNS isolates, stratified by treatment, are summarized in Appendix Tables 1.1, 1.2. In addition, Appendix Tables 1.3–1.10 summarize the MIC distribution of *Staphylococcus aureus*, *Staphylococcus* spp. (CNS), *Streptococcus* spp., and *Escherichia coli,* stratified by season.

3.3. Changes in resistance of isolates between dry off and post-calving

Table 5 compares the AMR patterns in CNS isolates at dry off (S1) and post-calving (S2) for cows that did or did

TABLE 1 Distribution of stratified random sample of milk bacterial isolates of dairy cows selected for antimicrobial susceptibility testing.

Organism type	Season		Sampling stage[a]			Treatment group		Total
	Winter	Summer	S1	S2	S3	Treated	Control	
Staphylococcus aureus	1	3	1	2	1	3	1	4
Coagulase negative Staphylococcus	176	245	241	161	19	210	211	421
Streptococcus spp.	10	27	13	19	5	15	22	37
Aerococcus spp.	2	6	4	3	1	6	2	8
Lactococcus spp.	2	4	5	1	0	3	3	6
Enterococcus spp.	1	2	2	0	1	2	1	3
Escherichia coli	5	14	6	12	1	6	13	19
Klebsiella	0	2	0	0	2	0	2	2
Corynebacterium spp.	4	13	10	7	0	4	13	17
Trueperella spp.	2	1	0	3	3	1	2	3
Bacillus spp.	13	10	11	10	2	15	8	23
Paenobacillus	1	0	1	0	0	1	0	1
Total	217	327	294	218	32	266	278	544

The selected isolates were stratified by season and sampling stage.
[a]Sampling stages included dry off (S1), post-calving (S2) and first mastitis event (S3).

TABLE 2 Antimicrobial susceptibility of mastitis bacterial isolates cultured from milk samples of dairy cows.

Organism type	N	Percent susceptibility against select antimicrobial drugs*									
		AMP	PEN	ERY	OXA	PIRL	P/N	TET	CEP	XNL	SDM
Staphyococcus aureus	4		100	100	100	100	100	100	100	100	0
Staphylococcus spp. (CNS)	421		72	94		95	98	95		99	28
Streptococcus spp.	37	100	100	94		97	100	83		93	
Coliforms (*E. coli* and *Klebsiella*)	21							57	44	100	30
Corynebacterium spp.	17	88	71					76	100	76	

Cows were enrolled over winter and summer seasons with milk samples collected from enrolled cows at dry off, post-calving and the first mastitis event within 150 days in milk.
*Ampicillin (AMP), penicillin (PEN), erythromycin (ERY), oxacillin (OXA), pirlimycin (PIRL), penicillin/novobiocin (P/N), tetracycline (TET), cephalothin (CEP), ceftiofur (XNL) and sulfadimethoxine (SDM). Grayed cells represent species drug combinations without MIC breakpoints.

not receive IMM AMD at dry off. Estimates for change in resistance of CNS against ampicillin, oxacillin and cephalothin were not assessed due to undefined CLSI MIC breakpoints (CLSI 2019; VET08, 4th ed). The greatest net difference in antimicrobial resistance (DAMR) against the tested AMDs, for CNS strains isolated from treated cows (AB or AB+ITS) between dry off and post-calving, showed a 12.2% increase in DAMR against penicillin, and a single negative DAMR for resistance to tetracycline (−4.9%). In contrast, the DAMR between S1 and S2 samples for CNS isolated from non-treated cows (ITS or control) for the same drugs, showed a greater increase in DAMR for penicillin (16.4%) and no change for tetracycline (0%). On the other hand, in addition to the 16.4% DAMR for penicillin in non-treated cows being the most increase, the only negative DAMR were for resistance to sulfadimethoxine (−6.1%) and ceftiofur (−2.0%).

3.4. Parametric survival interval regression models

Parametric survival interval regression models were limited to cows ($n = 86$) with CNS species isolates ($n = 172$) from samples collected at both dry off and post-calving due to the low frequency of the other species isolates (0 to 37 isolates). The Weibull distribution was the best fitting for all study models. Models for AMR against penicillin/novobiocin for cows that had CNS at dry off and post-calving were not specified since CNS isolates from 85 of the 86 cows were susceptible to penicillin/novobiocin at $\leq 1\,\mu g/ml$ and only a single isolate showed resistance at $8\,\mu g/ml$. Similarly, a model for post-calving CNS isolates' resistance against sulfadimethoxine couldn't be specified reliably due to extreme right censoring (22 of 23 isolates resistant or

TABLE 3 Percent of susceptible isolates ($n = 176$) by minimum inhibitory concentration (MIC; μg/ml) for *Staphylococcus* spp. (CNS) isolated from milk samples collected from dairy cows during fall/winter season.

	Percent *Staphylococcus* spp. isolates inhibited at different drug concentrations*													MIC 50	MIC 90
Drug concentration (μg/ml)	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>256		
Ampicillin	74	10	5	2	2	3	3	1						≤0.12	2
Penicillin	73	**9**	3	3	0	2	3	7						≤0.12	16
Erythromycin		41	46	3	1	1	**9**							1	1
Oxacillin					95	1	4							2	2
Pirlimycin			87	9	1	**1**	3							1	1
Penicillin/Novobiocin				98	0	**1**	0	2						1	1
Tetracycline				89	5	1	1	4						1	1
Cephalothin					95	2	1	0	2					2	2
Ceftiofur			60	31	7	1	**2**							1	2
Sulfadimethoxine									25	1	1	1	**73**	>256	>256

Milk samples were collected from enrolled cows at dry off, post-calving and the first mastitis event within 150 days in milk.
*Bold and underlined estimates signify isolate frequency resistant at the MIC cutoff for the respective drugs (CLSI 2019; VET08, 4th ed). Gray cells represent absence of the respective drug concentration on the plate (CMV1AMAF®; Sensititre®, Thermofisher).

TABLE 4 Percent of susceptible isolates ($n = 245$) by minimum inhibitory concentration (MIC; μg/ml) for *Staphylococcus* spp. (CNS) isolated from milk samples collected from dairy cows during spring/summer season.

	Percent *Staphylococcus* spp. isolates inhibited at different drug concentrations*													MIC 50	MIC 90
Drug concentration (μg/ml)	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>256		
Ampicillin	73	9	3	3	3	1	2	5						≤0.12	2
Penicillin	72	**9**	2	2	1	1	2	9						≤0.12	16
Erythromycin		33	57	4	1	2	**4**							1	1
Oxacillin					96	1	3							2	2
Pirlimycin			87	6	3	**1**	4							1	1
Penicillin/Novobiocin				98	0	**0**	1	1						1	1
Tetracycline				93	2	0	0	4						1	1
Cephalothin					96	0	1	1	1					2	2
Ceftiofur			43	44	10	1	**1**							1	2
Sulfadimethoxine									27	1	2	0	**71**	>256	>256

Milk samples were collected from enrolled cows at dry off, post-calving and the first mastitis event within 150 days in milk.
*Bold and underlined estimates signify isolate frequency resistant at the MIC cutoff for the respective drugs (CLSI 2019; VET08, 4th ed). Gray cells represent absence of the respective drug concentration on the plate (CMV1AMAF®; Sensititre®, Thermofisher).

95.7%) resulting in estimates greater than the logical drug concentration (MIC 10^6 μg/ml).

Final models for resistance in CNS against different AMD are summarized in Tables 6–8 and their predictions by treatment status are presented in Table 9. Model predictions represent the MIC (μg/ml) estimates for the CNS isolates at S2 for each drug.

Models for resistance of CNS post-calving to oxacillin, tetracycline, cephalothin and ceftiofur showed positive associations with exposure to dry off IMM therapy using AMD from the same drug class, a class with a similar resistance mechanism, or any AMD at dry off. In contrast, models for resistance of CNS to penicillin post-calving identified a negative association with exposure to any AMD at dry off. There were no associations between any dry off IMM AMD therapy and CNS resistance post-calving against the remaining drugs (ampicillin, pirlimycin or erythromycin).

Several cow-related factors were predictive of CNS isolate AMD at S2. Specifically, history of previous mastitis in the dry off lactation was associated with a decrease in resistance to penicillin, oxacillin and pirlimycin post-calving, in comparison to cows with no history of mastitis. In addition, a teat-end score four in any of the four quarters at dry off was associated with increased resistance of CNS to penicillin post-calving. However, CNS isolates from cows with udder hygiene score >2 at dry off had lower resistance to penicillin post-calving compared to cows with cleaner udders (lower hygiene scores). Cows with CMT score of three at dry-off had significantly lower resistance against ampicillin compared to

TABLE 5 Effect of antimicrobial therapy on change in the resistance of Coagulase Negative *Staphylococcus* (CNS) isolated at dry off and post-calving.

Anti-microbial agents	No Antimicrobial therapy at dry off							Antimicrobial therapy at dry off						
	S1 ($N^a = 49$)			S2 ($N^a = 49$)			DAMR	S1 ($N^a = 41$)			S2 ($N^a = 41$)			DAMR
	n	%	95% CI	*n*	%	95% CI	(%)	*n*	%	95% CI	*n*	%	95% CI	(%)
Penicillin	13	26.5	(15.96–40.72)	21	42.9	(29.71–57.10)	16.4	9	21.9	(11.73–37.31)	14	34.1	(21.23–49.94)	12.2
Erythromycin	3	6.1	(1.96–17.57)	5	10.2	(4.26–22.50)	4.1	0	0.0		3	7.3	(2.34–20.63)	7.3
Pirlimycin	2	4.1	(1.00–15.16)	2	4.1	(1.00–15.16)	0.0	0	0.0		3	7.3	(2.34–20.63)	7.3
Pen/Novo[b]	1	2.0	(0.20–13.43)	1	2.0	(0.20–13.43)	0.0	0	0.0		1	2.4	(0.33–0.15.75)	2.4
Tetracycline	2	4.1	(1.00–15.16)	2	4.1	(1.00–15.16)	0.0	4	9.8	(3.66–23.53)	2	4.9	(1.19–17.80)	−4.9
Ceftiofur	1	2.0	(0.20–13.4)	0	0.0		−2.0	0	0.0		1	2.4	(0.33–0.15.75)	2.4
SDMS[c]	38	77.5	(63.63–87.21)	35	71.4	(57.15–82.41)	−6.1	29	70.7	(54.99–82.70)	32	78.0	(62.69–88.27)	7.3

Difference in antimicrobial resistance (DAMR) for each antimicrobial drug was estimated as the change in percentage of resistant isolates between dry off (S1) and post-calving (S2). The change in resistance was evaluated for cows stratified by exposure to intramammary antibiotics. Antimicrobial drugs without established MIC breakpoints (Ampicillin, Oxacillin and Cephalothin) were excluded.

[a]N is the total of CNS isolates at each sampling stage; n is the number of isolates resistant to specific antibiotics.

[b]Penicillin/Novobiocin.

[c]Sulfadimethoxine.

cows with lower scores. Higher parity (>3 lactation) was associated with significant increase in CNS resistance to oxacillin compared to lower parity.

Region was only predictive of resistance of CNS to penicillin and ceftiofur post-calving; isolates from study cows in the NSJV herds showed less resistance than their counterparts in the GSCA herds. Seasonal changes were also observed, where CNS resistance to ceftiofur post-calving was higher in the summer compared to winter. Interestingly, after adjusting to dry off treatment, management, and cow factors, resistance at dry off was not predictive of resistance of CNS isolates post-calving to the same AMD across all models. Model predictions of MIC of CNS isolates at S2, by dry-off treatment status and difference between treated and untreated cows are summarized in Table 9.

4. Discussion

Coagulase negative *Staphylococcus* spp. (CNS) was the most common bacterial type isolated from milk samples collected across all the sampling stages and seasons of a previously described dry cow therapy trial in California. Similarly, other recent studies have reported CNS as the most common mastitis isolate in many regions (20–22). *Streptococcus* spp. and coliforms were the second and third most common isolates, respectively, with relatively lower frequencies compared to CNS. Coliforms are mainly associated with clinical environmental mastitis and are thus expected to occur at low frequency in non-clinical cows (23).

Overall, our results showed high susceptibility of CNS isolated from milk to common AMD used for mastitis therapy in dairy cows, in addition to other antimicrobial drugs included in a commercially available mastitis antimicrobial sensitivity testing plate. Similar results were reported in the US (24) and Canadian herds (25). In contrast to high susceptibility of isolates from North American and European countries, study reports from other continents have indicated high prevalence of resistance of mastitis pathogens against commonly used AMD. A recent study from China reported up to 64 and 34% resistance of CNS isolates from large Chinese dairy herds to penicillin and tetracycline, respectively, compared to the corresponding 28 and 5% resistance estimated in this study (26). Similarly, high resistance of CNS and others mastitis pathogens were reported in Ethiopia (27, 28), Jordan (29), and Brazil (30, 31). The finding of high AMR against common mastitis drugs correlates to the general pattern of bacterial resistance in these countries (28, 32, 33).

Despite the low level of resistance reported in this study, the results provide evidence of an association between IMM antimicrobial therapy at dry off and increased resistance of isolates recovered post-calving. Such a finding is an impetus for the development and implementation of stewardship programs that promote judicious use of AMD for mastitis therapy and hence maintain the low resistance status quo. In the US, approximately 93% of dairy cows are treated with AMD at dry off on 80.3% of the dairy herds (2). The current and long standing practice for control of bovine mastitis is to administer IMM antimicrobial infusion in all four quarters of all cows at dry off (blanket dry cow therapy) (34–36). However, recent studies have shown that selective therapy does not have a negative effect on cow health and performance during early lactation when compared to blanket dry cow therapy (11, 37–39). A judicious approach to AMD use would therefore involve identifying only high-risk cows to receive IMM AMD therapy at dry-off, as opposed to blanket therapy. Extension and outreach plans should be implemented to increase the awareness of the stakeholders, including dairy producers and veterinarians, on the development of AMR due to AMD therapy at dry off and strategic approaches for implementation of selective dry cow therapy programs.

The study data showed an association between resistance of post-calving CNS isolates to oxacillin, cephalothin, ceftiofur, and tetracycline, and dry off exposure to AMD from the same drug

TABLE 6 Final parametric survival interval regression models for penicillin, ampicillin, and oxacillin resistance in *Staphylococccus* spp. (Coagulase Negative *Staphylococcus*) isolated post-calving.

Tested drug	Variables	Levels	Coefficient	SE	*P*-value	MIC ratio	95% CI
Penicillin	IMM infusion at dry off[a]	No treatment	Referent				
		Treatment	−3.65	1.792	0.04	0.03	(0.0008, 0.87)
	Region	Southern SJV	Referent				
		Northern SJV	−6.64	3.250	0.04	0.001	(2.23e-06, 0.76)
	Interaction (IMM infusion at dry off X Region)[b]		8.35	3.336	0.01		
	Mastitis during dry off lactation	No	Referent				
		Yes	−3.75	1.899	0.04	0.02	(0.0006, 0.97)
	Udder hygiene score	≤ 2	Referent				
		> 2	−4.79	2.380	0.04	0.008	(7.83e-05, 0.88)
	Teat end score	< 4	Referent				
		4 at any teat	6.95	2.914	0.01	1048.23	(3.47, 316, 591.4)
	Intercept		1.12	1.663	0.50	3.06	(0.12, 79.76)
Ampicillin	IMM infusion at dry off[a]	No treatment	Referent				
		Treatment	−0.73	1.293	0.57	0.48	(0.04, 6.09)
	California mastitis test score at dry off	< 3	Referent				
		= 3	−3.14	1.542	0.04	0.04	(0.002, 0.88)
	Intercept		−2.08	1.664	0.21	0.12	(0.004, 3.26)
Oxacillin	IMM infusion at dry off	No treatment	Referent				
		Treatment with cloxacillin	1.83	0.144	< 0.01	6.21	(4.68, 8.23)
		Treatment other than cloxacillin	0.60	0.434	0.16	1.83	(0.78, 4.28)
	Mastitis during dry off lactation	No	Referent				
		Yes	−3.23	0.065	< 0.01	0.04	(0.03, 0.04)
	Parity	= 2	Referent				
		> 2	3.27	0.062	< 0.01	26.26	(23.25, 29.66)
	California mastitis test score at dry off	< 3	Referent				
		= 3	0.97	0.702	0.16	2.63	(0.66, 10.43)
	Intercept		−4.03	0.182	< 0.01	0.02	(0.01, 0.03)

IMM, Intramammary.

[a]Any antimicrobial drug (AMD) therapy: cloxacillin benzathine (Dryclox®, Boehringer Ingelheim), ceftiofur hydrochloride (Spectramast DC®, Zoetis), cephapirin benzathine (ToMORROW®, Boehringer Ingelheim), and combination of procaine penicillin G and dihydrostreptomycin (Quartermaster®, WG Critical Care, LLC).

[b]MIC ratio estimate comparing post-calving AMR against penicillin in CNS isolates from cows treated at dry-off vs. those untreated = 110.1 (SE 242.87); 95% CI 0, 586.2; P-value 0.65.

classes. Resistance associated with AMD therapies from the same drug class could be explained by the fact that bacterial organisms share common mechanisms of resistance to beta-lactam AMD, which include modifications of the drug target, penicillin binding proteins (PBP), or by producing the protective beta-lactamase enzymes. While chromosomal beta-lactamase are species-specific, the plasmid-mediated enzymes are transferrable between bacterial species and genera (40–43).

In contrast, the observed negative association between exposure to AMD at dry off that contained penicillin and penicillin resistance in CNS isolates post-calving. Our finding is in contrast to penicillin administration and

TABLE 7 Final parametric survival interval regression models for pirlimycin, erythromycin and tetracycline resistance in *Staphylococccus* spp. (Coagulase Negative *Staphylococcus*) isolated post-calving.

Tested drug	Variables	Levels	Coefficient	SE	*P*-value	MIC ratio	95% CI
Pirlimycin	IMM infusion at dry off[a]	No treatment	Referent				
		Treatment	0.21	1.354	0.87	1.23	(0.09, 17.50)
	Parity	= 2	Referent				
		> 2	3.35	1.467	0.02	28.43	(1.61, 503.69)
	Breed	Pure breed	Referent				
		Mixed breed	−9.85	2.241	< 0.01	5.29e-05	(3.94e-07, 0.006)
	Mastitis during dry off lactation	No	Referent				
		Yes	−9.76	2.420	< 0.01	5.78e-05	(5.94e-07, 0.004)
	Intercept		−5.53	2.008	< 0.01	0.003	(7.73e-05, 0.20)
Erythromycin	IMM infusion at dry off[a]	No treatment	Referent				
		Treatment	0.009	0.432	0.98	1.01	(0.43, 2.35)
	Intercept		−0.44	0.312	0.16	0.65	(0.35, 1.19)
Tetracycline	IMM infusion at dry off[a]	No treatment	Referent				
		Treatment	3.23	0.399	< 0.01	25.24	(11.55, 55.16)
	Intercept		−7.87	2.617	< 0.01	3.83e-04	(2.27e-06, 0.06)

AMD, Antimicrobial drug; IMM, Intramammary.
[a]Any antimicrobial drug (AMD) therapy: cloxacillin benzathine (Dryclox®, Boehringer Ingelheim), ceftiofur hydrochloride (Spectramast DC®, Zoetis), cephapirin benzathine (ToMORROW®, Boehringer Ingelheim), and combination of procaine penicillin G and dihydrostreptomycin (Quartermaster®, WG Critical Care, LLC).

TABLE 8 Final parametric survival interval regression models for cephalothin and ceftiofur resistance in *Staphylococccus* spp. (Coagulase Negative *Staphylococcus*) isolated post-calving.

Tested drug	Variables	Levels	Coefficient	SE	*P*-value	MIC ratio	95% CI
Ceftiofur	IMM infusion at dry off	No treatment	Referent				
		Treatment[a]	0.29	0.114	0.01	1.33	(1.06, 1.66)
	Season	Winter	Referent				
		Summer	0.37	0.185	0.04	1.44	(1.00, 2.07)
	Breed	Pure breed	Referent				
		Mixed breed	0.34	0.030	< 0.01	1.41	(1.33, 1.50)
	Region	Southern SJV	Referent				
		Northern SJV	−0.31	0.116	< 0.01	0.73	(0.58, 0.92)
	Intercept		−0.58	0.114	<0.01	0.56	(0.45, 0.70)
Cephalothin	IMM infusion at dry off	No treatment	Referent				
		Treatment with cephalosporins	6.43	0.640	< 0.01	620.83	(177.21, 2,175.04)
		Treatment other than cephalosporin	7.10	1.031	<0.01	1214.22	(160.91, 9162.45)
	Intercept		−9.35	1.285	< 0.01	8.67e-05	(<0.0001, 0.001)

IMM, Intramammary.
[a]Any antimicrobial drug (AMD) therapy: cloxacillin benzathine (Dryclox®, Boehringer Ingelheim), ceftiofur hydrochloride (Spectramast DC®, Zoetis), cephapirin benzathine (ToMORROW®, Boehringer Ingelheim), and combination of procaine penicillin G and dihydrostreptomycin (Quartermaster®, WG Critical Care, LLC).

resistance to penicillin and ampicillin previously observed in bovine mastitis *Staphylococcus aureus* isolates on Canadian dairy farms (44). The reason for the negative association between penicillin exposure at dry off and reduction in resistance against penicillin in CNS isolates post-calving is not known.

TABLE 9 Parametric survival interval regression model predicted Minimum Inhibitory Concentration (MIC) for *Staphylococccus* spp. (Coagulase Negative *Staphylococcus*) isolated post-calving from dairy cows by dry off antimicrobial drug (AMD) treatment status.

Model predicting AMR against:	Dry off AMD	Treated group		Non treated group		MIC Difference		*P*-value
		Coefficient	SE	Coefficient	SE	Estimate	SE	
Penicillin (treatment by region interaction)	Any AMD[a] (S. SJV)	0.08	0.081	3.06	5.094	−2.98	5.08	0.55
	Any AMD (N. SJV)	0.44	0.248	0.004	0.009	0.44	0.25	0.07
Ampicillin	Any	0.06	0.032	0.12	0.208	−0.06	0.184	0.72
Oxacillin	Cloxacillin	0.11	0.007	0.02	0.003	0.09	0.005	<0.01
	Treatment other than cloxacillin	0.03	0.013	0.02	0.003	0.015	0.014	0.28
Cephalothin	Cephalosporins	0.05	0.054	<0.01	<0.01	0.05	0.054	0.32
	Cephalosporins	0.11	0.048	<0.01	<0.01	0.11	0.047	0.02
Ceftiofur	Any	0.74	0.144	0.56	0.063	0.18	0.096	0.05
Pirlamycin	Any	0.005	0.007	0.004	0.007	<0.01	0.005	0.86
Tetracycline	Any	0.01	0.023	<0.01	0.001	0.01	0.022	0.67
Erythromycin	Any	0.64	0.286	0.65	0.201	0.01	0.281	0.98

[a]Any antimicrobial drug (AMD) therapy: cloxacillin benzathine (Dryclox®, Boehringer Ingelheim), ceftiofur hydrochloride (Spectramast DC®, Zoetis), cephapirin benzathine (ToMORROW®, Boehringer Ingelheim), and combination of procaine penicillin G and dihydrostreptomycin (Quartermaster®, WG Critical Care, LLC).

Interestingly, resistance of CNS isolated at dry off was not predictive of resistance post-calving which could be due to sample size. Our results also showed that treatment of cows with any of the AMD used for dry-cow therapy on the study herds resulted in an increase in resistance of CNS isolates to tetracycline post-calving. Most drugs induce selection and/or overexpression of multidrug efflux pumps which contributes to antimicrobial resistance (45). Since drug efflux is a major mechanism of resistance to tetracyclines, any drug that augments this process would potentially cause associated resistance to tetracycline (46–48). In addition, co-resistance to tetracycline and other AMD such as ampicillin, erythromycin, chloramphenicol, streptomycin, neomycin, gentamicin, sulfamethoxazole/trimethoprim was previously reported in staphylococcal isolates from domestic animals (49).

The main limitation of the current study was the small number of Gram positive or Gram negative bacteria isolated from milk samples. Fewer cows had the same species isolated at S1 and S2 making it difficult to compare the effect of the AMD on resistance in the same species. The current study also speciated non Staph isolates. As a result, CNS species were not identified and hence could differ between sampling points. Hence, the observed changes in the MIC values could be due to heterogeneity in AMR associated with different CNS species. In addition, further research is needed to estimate the effect of AMD IMM infusion on the development of AMR in mastitis pathogens other than CNS.

In conclusion, the current study showed low resistance of mastitis pathogens to AMD commonly used for mastitis therapy. However, the study provided evidence that IMM administration of AMD at dry off was associated with an increase in the AMR of CNS isolates post-calving. As such, antimicrobial stewardship on dairies including selection of cows for AMD administration at dry off should be guided by post-calving mastitis risk. Development and validation of a rapid, low cost and effective selective dry-cow therapy algorithm is required on dairy herds to improve antimicrobial stewardship.

Data availability statement

The datasets presented in this article are not readily available because data collected for this study is protected under California Food and Agriculture Code 14407. Requests for raw data may be made to the authors, who will consult with the California Department of Food and Agriculture (CDFA) and study producers on fulfilling the request. Requests to access the datasets should be directed to SA, saly@ucdavis.edu.

Author contributions

Conceptualization and funding acquisition: SA, TL, and EO. Methodology and investigation: SA, EO, WE, and DW. Software, supervision, and project administration: SA. Validation and writing original draft preparation: SA, EO, and WE. Formal analysis and data curation: SA and WE. Writing—review and editing: SA, EO, WE, DW, and TL. Visualization: EO. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Herd and animal level seroprevalence and associated risk factors of small ruminant brucellosis in the Korahey zone, Somali regional state, eastern Ethiopia

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Introduction: Brucellosis is a zoonosis of major public health and economic importance that is endemic in livestock in Ethiopia with varying levels of seroprevalence.

Methods: A cross-sectional study was carried out to determine the individual and herd-level seroprevalence of brucellosis in small ruminants in the Korahey zone of the Ethiopian Somali Region. A total of 324 sera from 63 herds of small ruminants were collected randomly using a multistage sampling technique and the sera were tested using the Rose Bengal Plate Test, and seropositive reactors were confirmed by the Complement Fixation Test.

Results and discussion: The seroprevalence of brucellosis at the herds and the individual level was 6.35% (95% CI: 0.0–13%) and 1.23% (95% CI: 0.0–2%), respectively; with 1.4% in goats and 0.9% in sheep. Moreover, predicted variables like age group, parity, history of abortion, fetal membranes, herd size, ownership of other livestock species, contact with wild animals in the past year, the introduction of new animals in the past year, and lending of breeding males in the past year were not significantly associated ($p > 0.05$) with *Brucella* seropositivity at individual and herd level seroprevalence during multivariable logistic regression analysis. Pastoral community awareness regarding the public health impact of brucellosis and the promotion of an intersectoral One Health approach for the effective control of brucellosis is recommended.

KEYWORDS

brucellosis, goat, risk factors, seroprevalence, sheep

1. Background

Brucellosis is a bacterial zoonotic disease caused by the genus *Brucella* that causes reproductive problems such as abortion, retained fetal membranes, and the birth of weak offspring, as well as orchitis and epididymitis in male animals, which is frequently followed by sterility (1). There are 12 known *Brucella* species that cause brucellosis at this time (2) and six of them, are known to be pathogenic to humans: *B. abortus*, *B. canis*, *B. inopinata*, *B. melitensis*,

B. pinnipedialis, and *B. suis* (2, 3). This taxonomic classification is primarily based on differences in host preference and pathogenicity that can be attributed to different proteomes, as demonstrated by specific outer-membrane protein markers (4, 5). Around the world, *Brucella* species that infect both humans and animals are frequently discovered in the interface of the human-animal ecosystem, where strong interactions exist between people, livestock, and wildlife in the same area (6, 7).

Brucella abortus causes abortion, stillbirth, and weak calves in cattle, with abortions typically occurring during the third trimester of pregnancy. *B. melitensis* is the most common species in developing countries in small ruminants and is linked to clinically visible diseases in humans (8). *B. melitensis* can cause abortion, retained placenta, orchitis, and epididymitis in goats. Abortions in goats are most common during the fourth month of pregnancy (9). Men's clinical manifestations of the disease include weakness, fever, excessive sweating, especially at night, weight loss, and generalized body aches (10). Swelling in the testes and burning micturition caused by orchitis and urethritis, respectively, are unusual symptoms of the disease in men (5, 11). The disease has also been reported in wild and marine mammals, as well as birds, in recent years. Another epidemiological concern is the presence of brucellosis in wild animals, with the potential for continuous transmission to domestic animals and from them to humans (12).

Prevalence studies for brucellosis have been carried out in various parts of the country. Brucellosis in animals and humans has been reported in various parts of Ethiopia, most notably in cattle in both intensive and extensive management systems (13–17). The disease has also been reported among small ruminants in pastoral areas of the country. In a study conducted in the Tallalak district of the Afar region, a prevalence of 13.7% in sheep and goats was reported, with the prevalence being higher in goats (15.4%) than in sheep (10.6%), as reported by Wedajo et al. (18), whereas in the Somali region, Mohammed et al. (19) reported a seroprevalence of 1.37% among small ruminants in three woredas of the Jigjiga zone.

Unpasteurized milk products, infected placental material, aborted fetuses, or infected animals, which can shed a variety of bacteria after abortion, are other ways in which this infection in humans can occur in endemic countries (20). Pastoralist communities are more likely to contract brucellosis and other zoonotic diseases than other communities that do not have the same level of association with animals due to their close physical proximity to livestock and their reliance on animal products (19, 21).

The prevalence of brucellosis in pastoralist areas is thought to be influenced by several variables, including grazing patterns, management techniques, and the age and sex composition of herds (22, 23). Additionally, for a variety of reasons, pastoralists do not isolate or get rid of animals that may be infected with Brucellosis, which raises the risk of transmission to healthy animals. Female sheep and goats are kept in the herds for a longer period than males and are sold as soon as they mature. Unlike males, infected females shed the bacteria more frequently and may contribute to the likelihood of brucellosis in pastoral areas. Other factors contribute to the prevalence of brucellosis in pastoralist areas, such as the consumption of unpasteurized milk; unsafe handling and improper disposal of potentially infective materials, such as aborted fetuses, fetal membranes, and bodily fluids, which may contain concentrations of the bacteria; and a lack of awareness about zoonotic risks and methods of transmission (4, 16, 17, 20).

Nonetheless, the extent of the disease and its impact on pastoralist health are understudied, at least in the context of the proposed study area: the Korahey zone. Similarly, no research has been done on the risk factors associated with disease occurrence or the existing knowledge, attitudes, and practices in pastoralist areas that may play a role in zoonotic transmission. Therefore, the objective of this study was to estimate seroprevalence and associated risk factors of brucellosis in small ruminants in selected districts of the Korahey zone, Somali regional state, Ethiopia.

2. Methods

2.1. Study area

The study was conducted in three purposively selected districts of the Korahey zone, namely, Doboweyn, Kebridahar, and Sheygosh, located 470, 380, and 280 km, respectively, south of Jigjiga, the capital of the Somali Region of Ethiopia, which is situated approximately 630 km to the east of Addis Ababa. The Korahey zone is located in the south of the region and consists of 10 districts and one city administration; the zonal capital is Kebridahar. The zone has semiarid agroecology with a bimodal rainfall pattern. The main rainy season is *Gu* (April–June), and the second rainy season, *Deyr,* is received between September and November. The major production system in the zone is pastoralism, with some mixed livestock crop production practiced around Kebridahar. The livestock population of the zone is 3,576,492, consisting of 311,243 cattle, 1,323,491 goats, 1,265,585 sheep and 582,860 camels (24). Kebridahar is located at 6°44′N latitude and 44°16′E longitude and has a total of 799,367 of livestock population. Doboweyn is located at 6°41′N latitude and 43° 69′E longitude and has a total of 626,674 of livestock population. Sheygosh is located at 7° 41' N latitude and 43° 56′ E longitude and has a total of 520,900 of livestock population (Figure 1).

2.2. Study population

There are approximately 1,323,491 goats and 1,265,585 sheep in the Korahey zone (25). The study population consisted of small ruminants kept under an extensive management system in three purposively selected districts of the Korahey zone. The study animals were indigenous Somali goats and black head Ogaden sheep. In addition, sheep and goats, which were above 6 months of age and had no history of vaccination against brucellosis, were included in the study.

2.3. Study design

A cross-sectional study design was used to estimate the seroprevalence and associated individual animal-level and herd

Abbreviations: BOFED, Bureau of Finance and Economic Development; CAT, Card agglutination test; CFT, Complement fixation test; CFSPH, Center for Food Security and Public Health; ELISA, Enzyme-linked immunosorbent assay; ICSP, International Committee on Systematics of Prokaryotes; LPDB, Livestock and Pastoralist Development Bureau; NVI, National Veterinary Institute; OIE, Office International des Epizooties; RBPT, Rose Bengal Plate Test; SRBC, Sensitized sheep red blood cells; SRS, Somali regional state; WHO, World Health Organization.

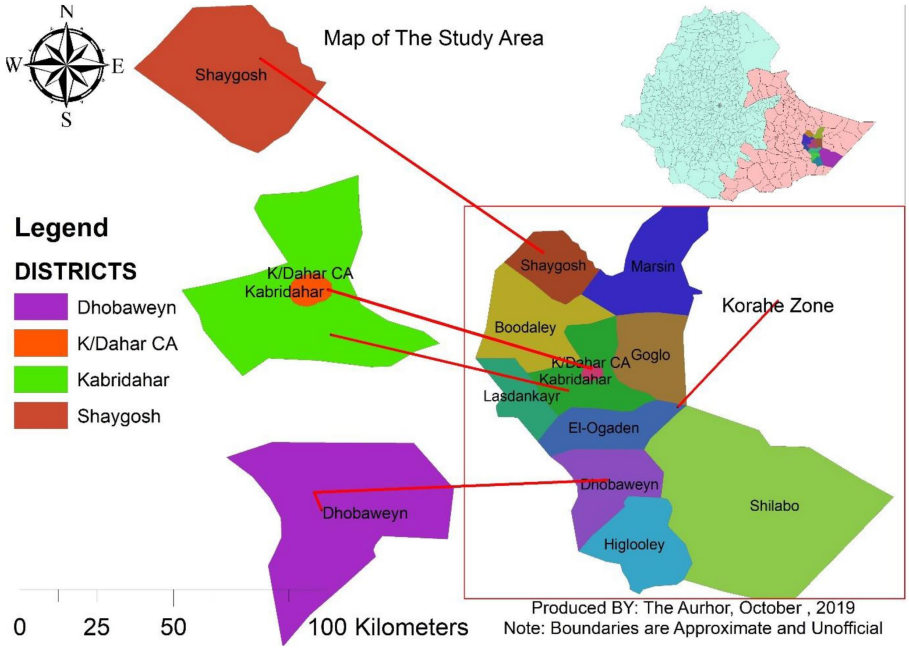

FIGURE 1
Map of the three study districts of the Korahey zone, Somali regional state, and eastern Ethiopia.

-level risk factors for small ruminant brucellosis and to evaluate the knowledge, attitudes and practices of pastoralists toward brucellosis in selected districts of the Korahey zone, Somali region of Ethiopia.

2.4. Sampling method

A multistage sampling technique was employed. The pastoralist association was the primary sample, pastoralist families/herds the secondary sample and the individual animals the tertiary sample. The primary sample was selected purposively based on livestock population and accessibility, whereas the secondary and tertiary samples, herds and individual animals, were selected using a systematic random sampling technique.

Individual animals were sampled from the herds above using a systematic random sampling method by first putting the animals in a crush. Then, the animals were allowed to leave the fence one animal at a time. The herd's owner was asked to randomly pick an animal from among the first 5, leaving the fence. Then, every k^{th} animal was selected; the value of k was determined based on the size of the herds being sampled and the number of animals sampled from each herd. Sheep and goats were sampled separately.

2.5. Sample size determination

The sample size was determined using Thrusfield (26) formula as follows:

$$n = \frac{1.96^2 \, Pexp\,(1 - Pexp)}{d^2}$$

where n = required sample size, Pexp = expected prevalence and d = desired absolute precision.

Accordingly, the estimated sample size was 23 for goats and 15 for sheep based on the expected brucellosis prevalence of 1.5% in goats and 1% in sheep in other parts of the Somali region by Mohammed et al. (19) and 0.05 desired absolute precision at the 95% level of confidence. However, to increase precision, the sample size was increased to 213 goats and 111 sheep. In total, 324 small ruminants (sheep and goats) were sampled using systematic random sampling (Table 1).

Animals were sampled from nine purposively selected kebeles that were located in three study districts, i.e., three kebeles (peasant associations) per woreda (districts) based on livestock population and accessibility. The distribution of the herds across the kebeles and woredas was determined based on the estimated population size of the respective kebeles.

2.6. Blood sample collection, transportation, and storage

Approximately 10 mL of blood sample was collected from the jugular vein of each study animal using plain vacutainer tubes, needle holders, and needles. The blood sample from each animal was labeled and centrifuged at 10,000 rpm for 3 min, and sera were removed by siphoning them into sterile cryovials. The serum samples were then transported to the Jigjiga regional veterinary diagnostic and research

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TABLE 1 Total number of small ruminants sampled from each kebele (peasant associations).

Woreda	Kebele	Total herds listed	Herds sampled	Animals sampled		
				Goat	Sheep	Total (% sampled)
Doboweyn	Doboweyn town 01 kebele	84	9	17	17	34 (10.5)
	Harano	112	9	20	14	34 (10.5)
	Jidhale	98	9	20	14	34 (10.5)
Kebridahar	01 Kebele, Kebridahar	71	9	23	13	36 (11.1)
	Bundada	131	9	33	9	42 (13)
	Dalad	117	9	26	8	34 (10.5)
Sheygosh	01 Kebele Sheygosh	66	9	19	19	38 (11.7)
	Harir	109	9	29	9	38 (11.7)
	Wijiwaji	96	9	26	8	34 (10.5)
	Total	884	63	213	111	324

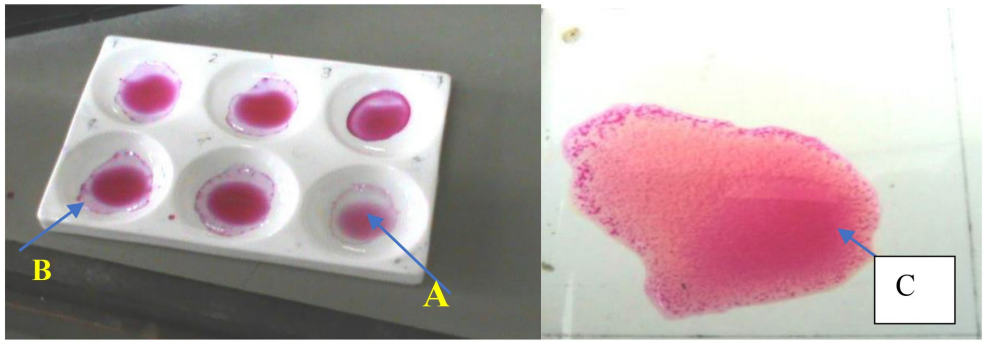

FIGURE 2
Plate Rose Bengal Plate Test showing positive and negative samples. A, positive sample; B, negative sample; C, positive control.

laboratory in an ice box, where they were stored and kept at −20°C until serology procedures were performed.

2.7. Serological tests

The screening procedure with RBPT was performed at Jigjiga Regional Veterinary Diagnostic and Investigation Laboratory and National Veterinary Institute (NVI) at Bishoftu, whereas CFT was conducted at NVI using test protocols as outlined by OIE (27) and the manufacturer's specifications for the tests. All the samples that tested positive on the RBPT were tested with the complement fixation test (CFT) (Figure 2).

The CFT and RBPT test antigens (*Brucella abortus* strain 99), control sera, and other reagents were obtained from Atlas Medical, William James House, Cowley Rd. Cambridge Cb4, 4 WX and sensitized sheep red blood cells (SRBCs) were obtained from the NVI. The serum specimens were tested serially first using RBPT and then CFT for those that tested positive for RBPT. An animal was considered positive if the serum specimen tested positive on both RBPT and CFT, whereas a herd was considered positive if at least a single serum specimen from an animal within the herds tested positive on both RBPT and CFT. For RBPT, the Rose Bengal test antigen was prepared from the killed standard strain of *B. abortus* strain 99 and stained with Rose Bengal dye in an acidic buffer pH 3.65.

2.8. Questionnaire survey

A pretested structured questionnaire was used to collect information about potential factors associated with brucellosis seropositivity at animal and herd levels. The potential factors at the individual animal level included age, parity stage, and previous history of reproductive problems such as abortion, retained fetal membranes, orchitis, and epididymitis. At the herd's level, the factors assessed were herd size, keeping other animals with sheep and goats, the introduction of new animals in the past year, abortion in the herds in the past year, contact with other small ruminant herds and wild animals, presence of calving/lambing/kidding pens, vaccination history and whether owners sought veterinary service or advice in the past year.

2.9. Data management and analysis

Serological data were entered into a Microsoft Excel Spreadsheet version 2016 (Microsoft Corporation) along with the corresponding

data generated with the questionnaire. The statistical software package SPSS version 20 (SPSS Inc., IBM, Chicago, IL, United States) was used for data analysis. Descriptive statistics like frequency and proportion were employed for the description of the seroprevalence of the disease. A herd level and individual animal seroprevalence were calculated by dividing the number of positive test results by the total number of herds and animals sampled, respectively. Univariable analysis using Fisher's exact test was used for the analysis of the association between individual animal-level and herds *Brucella* seropositivity and the potential factors. Furthermore, a multivariable logistic regression model was used to analyze risk factors of the disease that was found statistically significant when using univariable analysis and the results were reported by odds ratio using 95% confidence interval to assess the strength of the association. The final multivariable logistic regression model selection was based on p value (p value $\leq$0.25) and stepwise backward elimination procedure, dropping the least significant independent variable until all the remaining predictor variables were significant. The statistical significance level was set at 95% confidence level and 5% level of precision so that $p \leq 0.05$ was considered significant.

3. Results

3.1. Herds characteristics

A total of 324 animals composed of 213 (65.7%) goats and 111 (34.3%) sheep were sampled. These animals were sampled from 63 herds from 9 kebeles (peasant associations) in Doboweyn, Kebridahar, and Sheygosh districts of the Korahey zone, Somali Region of Ethiopia. The 63 herds had a total of 4,026 animals, of which 2,512 were goats and 1,514 were sheep. The median sheep and goat herds' size was 56 (range: 20–180 animals). One-third of the herds in the study (33.3%) had more than 60 heads of sheep and goats, and the remaining 66.7% had a herd size of 60 heads or below.

3.2. Seroprevalence of brucellosis at the individual animal and herd levels

3.2.1. *Brucella* seroprevalence at the individual animal level by animal species

Of the 324 serum samples examined, 1.85% (95% CI: 0.0–3.0%) were positive for *Brucella* antibodies on RBPT. Of the samples positive for RBPT, 2.3% (95% CI: 0.0–4%) were from goats, and 0.9% (95% CI: 0–3%) were from sheep. Of the samples testing positive on RBPT, 1.23% (95% CI: 0.0–2%) tested positive on CFT, of which 1.4% (95% CI: 0.0–3%) were goats and 0.9% (95% CI: 0–3%) were sheep. In this study, the animal-based seroprevalence of small ruminants brucellosis in three district of the Korahey zone of Somali regional state was 1.85% by RBPT and 1.23% by combined RBPT and CFT. Thus, the overall seroprevalence was 1.23% and were taken for subsequent data analyses (Table 2).

3.2.2. *Brucella* seroprevalence at the individual animal level by sublocation sampled

Based on CFT, the highest seroprevalence of 5.9% (95% CI: 2–14%) was recorded in the Dalad kebele of Kebridahar district, followed by 2.9% (95% CI: 3–9%) in the Harano kebele of Doboweyn district and then by 2.6% (95% CI: 3–8%) in the 01 kebele in Sheygosh town of Sheygosh district. In the remaining 6 locations, namely, 01 kebele of Kebridahar town and Bundada kebele of Kebridahar district; 01 kebele of Doboweyn town and Jidhale kebele of Doboweyn district and Harir and Wijiwaji kebeles of Sheygosh district, zero seroprevalences were recorded.

3.2.3. *Brucella* seroprevalence at the herd level

Of the 63 herds sampled, the overall true herd seroprevalence was 6.35% (95% CI: 0.0–13%). None of the herds had more than one animal testing positive for *Brucella* species. All the herds sampled had a mixture of sheep and goats; hence, comparing herds' seropositivity levels by species was deemed irrelevant.

3.3. Factors associated with brucellosis seropositivity at individual and herd levels

3.3.1. Bivariate analysis for individual-level factors associated with brucellosis in sheep and goats

In determining the risk factors associated with individual animal *Brucella* seropositivity, several factors were examined in the bivariate analysis. These include species, sex, age, number of parties, previous history of abortion and/or retained fetal membrane, and previous history of orchitis and epididymitis.

In terms of sex, 276 (85.2%) were females, and 48 (14.8%) were males. All the seropositive animals were females, but there was no statistically significant difference in the brucellosis seroprevalence between the two sexes ($p > 0.05$).

The majority, 196/324 (60.5%), of the sampled animals were between 3 and 4 years old, and only 18 (5.5%) were older than 4 years. A significant difference ($p = 0.001$) was observed between the different age categories with regard to *Brucella* seropositivity, with the highest prevalence being in the >4-year-old age category (16.7%), followed by the category aged 3–4 years (0.5%). None of the animals in the age range of 1–2 years tested positive for *Brucella*.

Of the female animals in the sample, 258/276 (93.4%) gave birth at least once, with 13 (4.7%) of them being primiparous, giving birth only once. Most of the female animals (84.8%) gave birth between 2 and 4 times. Only 11 females (4%) gave birth more than four times. The highest seroprevalence was observed in the category of female animals that gave birth more than 4 times, with 3 of the 11 female animals in this category (27.3%) showing seropositivity with CFT. This was followed by the females that gave birth between 3 and 4 times, in which a seropositivity prevalence of 0.6% was recorded.

A quarter, 69/276 (25%), of the female animals sampled had a history of abortion at least once in the past, whereas 49/276 (17.8%) of female animals experienced retained fetal membranes. Regarding male animals, 11/48 (23%) had a history of experiencing either orchitis or epididymitis, but this study did not find any seropositive males. All the seropositive animals had a previous history of abortion and retained fetal membranes, and it was found that there was a significant difference ($p < 0.05$) between animals with a history of previous abortion and those without a previous history of abortion (Table 3).

TABLE 2 Seroprevalence of *Brucella* antibodies by test type and species.

Test	SPECIES	No of examined animals	No. of positive sampled	Percentage (%)	95% CI
RBPT	SHEEP	111	1	0.9	0.0–3.0
	GOAT	213	5	2.3	0.0–4.0
	TOTAL	324	6	1.85	0.0–3.0
CFT	SHEEP	111	1	0.9	0.0–3.0
	GOAT	213	3	1.4	0.0–3.0
	TOTAL	324	4	1.23	0.0–2.0

TABLE 3 Univariate analysis of seropositivity of *Brucella* with associated risk factors at individual animal level ($n = 324$).

Variable	Category	*Brucella* seropositivity n (%)	Negative n (%)	χ^2	*p*
Species	Goats	3 (1.4)	210 (98.6)		0.69
	Sheep	1 (0.9)	110 (99.1)	0.154	
Sex	Female	4 (1.4)	272 (98.6)		0.401
	Male	0	48 (100)	0.704	
Age category	≤2 years	0	110 (100)		<0.0001*
	3–4 years	1 (0.5)	195 (99.5)	37.375	
	>4 years	3 (16.7)	15 (83.3)		
Number of parity	0–2 times	0	102 (100)		<0.0001*
	3–4 times	1 (0.6)	162 (99.4)	53.655	
	5–6 times	3 (27.3)	8 (72.7)		
Previous history of abortion	Yes	4 (5.8)	65 (94.2)	14.967	0.001*
	No	0	207 (100)		
Retained fetal membrane	Yes	4 (8.2)	45 (91.8)	22.730	<0.0001*
	No	0	227 (100)		
Orchitis and epididymitis	Yes	0	11 (100)	0.704	0.703
	No	0	37 (100)		

*Indicates variables with a statistically significant values. OR, odds ratio; CI, confidence interval.

3.3.2. Bivariate analysis of seropositivity of *Brucella* with herd-level risk factors in sheep and goats

In determining the risk factors associated with herds *Brucella* seropositivity, several factors were examined in the bivariate analysis. These included herds' size, ownership of other livestock species, ownership of calving/kidding/lambing pens, contact with other herds and contact with wildlife, history of abortion in the herds, the introduction of new animals, lending/borrowing of male animals, and veterinary service-seeking behavior of the owner. All but the last factor, i.e., veterinary service-seeking behavior, was considered a risk factor, while the latter variable was treated as a protective factor.

One-third of the herds (21/63, 33.3%) had more than 60 heads of sheep and goats, and the remaining herds had a maximum herd size of 60 sheep and goats. The herds with a size of more than 60 animals had almost 7 times higher odds of having positive reactors for *Brucella* compared to those herds with smaller herds sizes, but this was not statistically significant (OR = 6.833; 95% CI: 0.665–70.235, $p = 0.106$).

Of the 63 sampled sheep and goat herds, 25 (39.7%) are kept together with other livestock species, as the owner also keeps other species of livestock apart from goats and sheep, and the other 38 (60.3%) households possess only small ruminants. Almost all of the respondents, 24/25 (96%), who also own other livestock species raise the different herds separately, and only one respondent said that they house the different species of animals together. Most of the other livestock species kept in the area are camels, cattle, and donkeys ordered in terms of population. Three of the herds with positive reactors to *Brucella* (75%) came from herds whose owners also keep other livestock species, and even though it is not statistically significant, sheep and goat herds belonging to owners who also keep other livestock species have 5 times more odds of getting brucellosis compared to other herds whose owners do not keep other livestock species as well (OR = 5.045; 95% CI: 0.494–51.540, $p = 0.172$).

Nearly all of the herds 62/63 (98.4%) did not have a calving/lambing/kidding pen where animals delivered offspring, and all the herds with positive reactors to *Brucella* were from those herds that lacked the calving pen, but this was not statistically significant ($p > 0.05$). Since the area is predominantly pastoralist with grazing lands shared communally, there is a high chance of contact between different herds. In the present study, 52/63 (82.5%) of the herds came

into contact with other herds of sheep and goats in the past year, but the current study did not find a statistically significant association between *Brucella* herds seropositivity and contact with other herds (OR = 0.612; 95% CI: 0.058–6.505, $p = 0.684$).

On the other hand, 14/63 (22%) of the herds came into contact with wild animals in the past year. The most commonly encountered wild animals were antelopes such as dik-dik, warthogs, foxes, and hyenas. Although it was not statistically significant, herds that came into contact with wild animals had a higher chance of having positive reactions to *Brucella* (OR = 3.917; 95% CI: 0.499–30.728, $p = 0.194$).

Abortion in sheep and goat herds was reported in 32/63 (50.8%) of the herds in the past year. Of the 4 herds that tested seropositive for *Brucella*, 3 (75%) had experienced abortion in the past year compared to 29 (49%) in seronegative herds. Herds with abortion reports in the past year had an approximately 3 times higher chance of containing seropositive reactors than herds that did not experience abortion in the past year, but this difference was not significant (OR = 3.103; 95% CI: 0.305–31.580, $p = 0.339$).

New animals were introduced into 14/63 (22.2%) of the herds in the past year mainly through purchases and gifts from relatives. Comparing herds that introduced a new animal into the herds in the past year to those herds that did not, the study found that herds that introduced new animals into the herds had 13 times increased chances of having seropositive reactors for *Brucella* infection (OR = 13.091; 95% CI: 1.241–138.11, $p = 0.032$).

Sharing of breeding males is less common, and only 13/63 (20.6%) of the herds have lent their breeding male sheep or goat animals over the last year. Herds that lend male animals for breeding purposes have a more than 14-fold increased chance of having seropositive reactors compared to herds that do not lend their male animals to other herds for breeding. This was statistically significant (OR = 14.7; 95% CI: 1.384–156.179, $p = 0.026$).

Although 26/63 (41.3%) of the herds were vaccinated in the past year, no vaccination was given against brucellosis, and only 19 (30%) respondents had received/obtained veterinary advice last year, with the majority, 44 (70%), not getting veterinary advice of any kind in the past year. No statistically significant difference ($p > 0.05$) in herds' seropositivity was observed between the herds in relation to vaccination and receiving veterinary advice (Table 4).

3.3.3. Multivariable analysis to determine independent factors associated with *Brucella* herds seropositivity

Based on the entry criteria stated in the methodology ($p \leq 0.25$), herds' size, ownership of other livestock species, contact with wild animals in the past year, the introduction of new animals in the past year, and lending of breeding males in the past year were selected for the multivariable analysis using a binary regression model. Additionally, age category, previous history of abortion, number of parity and retained fetal membrane history were selected for multivariable regression model from individual seropositive animal level factors. Nevertheless, none of the variables showed a significant association ($p > 0.05$) in the multivariable analysis (Table 5).

4. Discussion

In this study, the animal-based seroprevalence of small ruminant brucellosis in three districts of the Korahey zone of Somali regional state was 1.23% and this was in line with the results reported by Mohammed et al. (19) in the Jigjiga zone of the Somali region, with a prevalence of 1.37%; Tsegay et al. (28) in Debrezeit and Modjo, with a seroprevalence of 1.76%; and Dabassa et al. (29), who found a similar result (1.56%) in a study of small ruminant brucellosis in Yabello. The present finding is lower than results reported by Aloto et al., (30) who reported 4.1% in two zones of southern Ethiopia, Dosa et al. (31) who reported 3.33% in two selected districts of Wolaita Zone southern region, Teshome et al. (32) who reported 17.36% in goats in Borana pastoral area, Deddefo et al. (33) who reported 4.6% in Arsi, Teshale et al. (34) who reported 9.7% in Afar, and Negash et al. (35) who reported 9.11% in the Dire Dawa area. Similarly, Wedajo et al. (18) reported a higher seroprevalence (13.7%) in the Tallalak district of the Afar region.

However, the current result is relatively higher than the previous study by Ferede et al. (36) who reported 0.4% in and around Bahirdar and Tewodros and Dawit (37) who reported 0.7% in and around Kombolcha, Amhara region. This variation might be caused by variations in sample size, agroecological location, and animal management practices.

In the present study, RBPT was used to detect the seroprevalence of *Brucella* species in small ruminants which was used in this study to screen individual animals, is a low-cost, quick, and highly sensitive test (27). However, due to cross-reactivity with antibodies from closely related gram-negative bacteria such as *Yersinia enterocolitica, Escherichia coli, Salmonella* spp., and *Sternotrophomonas maltophilia* as well as antibodies produced by the *B. abortus* S19 vaccine, its specificity is low (38). In the current study, only samples that gave signals for both RBPT and CFT were considered positive since no single test is appropriate in all epidemiological situations due to problems of sensitivity and or specificity of the tests as recommended by OIE (27) and other reports (39).

The current study revealed that the seroprevalence of brucellosis was 1.57 times higher than sheep's seroprevalence, although this difference was not statistically significant. This finding is comparable to that of Mohammed et al. (19), who also reported higher seroprevalence in goats in the Somali region. Similar findings were also reported by Wedajo et al. (18), Teshale et al. (34), and Ashenafi et al. (40) in the Afar region; Aloto et al. (30) in two zones of southern, Ethiopia; Mengistu (41) in Konso, southern Ethiopia; Tewodros and Dawit (37) in Kombolcha of the Amhara region; and Negash et al. (35) in Dire Dawa.

However, a study by Bekele and Kasali (42) in the central highlands of Ethiopia and Samaha et al. (43) in Egypt showed a higher prevalence in sheep than in goats, mainly due to differences in husbandry systems and the susceptibility of the sheep and goat breeds in the particular area. The difference may also be due to variations in the species breakdown of the samples examined by the various researchers. Because goats are more susceptible to *Brucella* infection than sheep and excrete the bacterium for a longer period of time, goats exhibit higher seroprevalence than sheep (21).

The seroprevalence of brucellosis of female sheep and goats were higher than males one, although this difference was not statistically significant ($p > 0.05$). The result of this study is in agreement with other findings by Tewodros and Dawit (37) and Yesuf et al. (44), who also reported higher seroprevalence in females than in males, although Yesuf et al. (44) found a statistically significant ($p < 0.05$) difference in *Brucella* seroprevalence between the two sexes. The lower number of males ($n = 48$) sampled and tested compared to a higher number of

TABLE 4 Univariate analysis comparison of factors associated with *Brucella* seropositivity among positive and negative herds.

Variable	Category	Herds positive, n (%)	Herds negative n (%)	OR (95% CI)	*p*
Herds' size	>60	3 (75)	18 (30.5)	6.833 (0.665–70.235)	0.106
	≤60	1 (25)	41 (69.5)		
Ownership of other livestock species	Yes	3 (75)	22 (37.3)	5.045 (0.494–51.540)	0.172
	No	1 (25)	37 (62.7)		
Contact other sheep and goat herds in the past year	Yes	3 (75)	49 (83)	0.612 (0.058–6.505)	0.684
	No	1 (25)	10 (17)		
Contact with wild animals	Yes	2 (50)	12 (20.3)		
	No	2 (50)	47 (79.7)	3.917 (0.499–30.728)	0.194
Abortion in sheep and goat herds in past year	Yes	3 (75)	29 (49)		
	No	1 (25)	30 (51)	3.103 (0.305–31.580)	0.339
Introduction of new sheep and goats	Yes	3 (75)	11 (18.6)	13.09 (1.241–138.11)	0.032*
	No	1 (25)	48 (81.4)		
Lend breeding male in past year	Yes	3 (75)	10 (17)	14.7 (1.384–156.179)	0.026*
	No	1 (25)	49 (83)		
Seek veterinary service in past year	Yes	2 (50)	17 (29)	0.405 (0.53–3.111)	0.385
	No	2 (50)	42 (71)		

*Indicates variables with a statistically significant values. OR, odds ratio; CI, confidence interval.

TABLE 5 Multivariable logistic regression analysis of the variables associated with herds and individual-level seropositivity for *Brucella* in sheep and goats.

Variable characteristics	OR	[95% CI]	S.E.	*p*
Herds size (>60 or ≤ 60)	5.307	0.237–118.695	1.586	0.292
Ownership of other livestock species (yes or no)	2.906	0.186–45.447	1.403	0.447
Contact with wild animals in past year (yes or no)	4.225	0.218–82.060	1.514	0.341
Introduction of new animals in past year (yes or no)	3.354	0.170–66.232	1.522	0.427
Lend breeding male in past year (yes or no)	13.398	0.714–251.590	1.496	0.083
Age category (≤2 years/3–4 years/>4 years)	18.271	0.256–1303.684	39.783	0.182
Number of parity (0–2 times, 3–4 times, 5–6 times)	24.936	0.482–1290.939	50.215	0.110
Previous history of abortion (yes or no)	4.127	2.231–19.021	1.091	0.651
Retained fetal membrane (yes or no)	2.349	0.346–12.902	1.439	0.498

OR, odds ratio; SE, standard error; CI, confidence interval.

females ($n = 276$) in the sample may have contributed to the higher female animal seropositivity. It is also possible that male animals are less likely to contract *Brucella* infection because they do not contain erythritol (45). The fact that there was no statistically significant difference between the two sexes may also have contributed to the very low number of positive results observed in the current study.

The study revealed a significantly ($p < 0.05$) higher seroprevalence of small ruminant brucellosis in the old age group than in the medium and young age groups. This is consistent with the findings of Tsegaye et al. (28) and Adugna et al. (46), who also reported a higher seroprevalence of brucellosis in small ruminants more than 2 years of age than in the younger age categories. Similarly, Megersa et al. (14), Mohammed et al. (19), and Tigist et al. (47) reported higher seroprevalence in older age groups than in younger animals, even though the difference was not statistically significant. In contrast, Wedajo et al. (18) and Negash et al. (35) reported a higher seroprevalence in younger animals than in adult sheep and goats.

The study also found a statistically significant association between seroprevalence and parity stage, with higher seropositivity in higher parity stage females than in lower parity stage females. The various findings regarding the variation in brucellosis seroprevalence among the various age groups may be related to variations in the relative proportion of the various age groups in the samples examined by the various researchers. The risk of contracting *Brucella* infection is higher in sexually mature and pregnant animals than in sexually immature animals of either sex. This might be due to the concentration of erythritol and sex hormones, which promote the growth and reproduction of *Brucella* species organisms, rising with age and sexual maturity (48).

In the present study, the relationship between *Brucella* seropositivity and the presence of reproductive issues, such as a history of abortion or retained fetal membranes, was investigated. Male animals of both species were excluded from this discussion because no male animals tested positive for *Brucella* infection by chance. The seroprevalence of brucellosis in small ruminants with a history of abortion or retained fetal membranes was found to be higher ($p < 0.05$) than in those without these problems. Similar findings were reported by Wedajo et al. (18) and Wubishet et al. (49) in the Afar and Guji zones of the Oromia region, respectively. It is

known that abortion in livestock represents the major complaint attributed to *Brucella* infections (50–52).

The seroprevalence of brucellosis was higher in large (>60 heads) herds' sizes than in small (≤60 heads), but the difference was not significant. This is in line with the findings reported by Wedajo et al. (18) in the Afar region. Walker (53) shows that herd sizes and animal densities are directly related to disease prevalence and complicate infection control in a population. Similarly, sheep and goat herds kept alongside other livestock species had higher brucellosis seroprevalence than herds kept solely, where the owner did not own any other livestock species besides sheep and goats.

The introduction of new animals from unscreened herds into sheep and goat herds was a major risk factor observed in this study. Pastoralists usually introduce these animals into the herds as replacement stock through purchases, gifts, or donations from relatives. This finding is consistent with the findings of several authors who discovered that the introduction of animals from non-free Brucellosis herds or herds with unknown Brucellosis status was a major factor associated with Brucellosis in sheep, goat, and cattle herds (50, 54–57).

Other research suggests that introducing infected animals can increase individual-level prevalence because the longer they are in contact with the rest of the herds, the greater the risk of spread (1, 58). Animal movement between herds has also been found to be a potentially dangerous practice. One suggested key preventive measure is to avoid the introduction of infected animals by maintaining completely closed herds or by carefully screening purchased animals before introducing them into the herds, a practice that is very uncommon in pastoral communities. There is evidence that one of the main causes of most brucellosis control campaigns' ineffectiveness is the lack of control over the movement of animals, and this suggestion is supported by available data (12, 50, 56).

Seroprevalence was higher in herds with female animals that had abortions in the previous year compared to herds without abortions, but this difference was not statistically significant. Tigist et al. (47) and Obonyo (59) revealed a statistically significant correlation between a herd's seropositivity to *Brucella* and the presence of female animals that had recently given birth. Brucellosis causes late-term abortions, which increases the risk of the disease spreading to other animals in the herds while they graze on the contaminated pasture lands because aborting animals typically shed the bacteria into the environment. Abortion represents the major complaint attributed to *Brucella* infections in livestock (4, 50–52, 60). It is known that females infected with brucellosis shed considerable amounts of the pathogen in milk, placental membranes, and aborted fetuses. Such females have been reported to shed organisms for several months (4, 48, 60). This causes environmental contamination, which increases the risk of pathogen transmission between animals in the same herds as well as other herds during free mixing in grazing and watering areas.

Lending or sharing male animals with other herds for natural breeding purposes was significantly associated with herd seropositivity. Other authors have reported that lending male animals for breeding is a risk factor for *Brucella* seropositivity in animals (59, 61). Although the venereal route is not regarded as a key channel for *Brucella* transmission in small ruminants under natural settings, procedures that entail the movement of animals between herds s are deemed problematic because of the possibility of mechanical transmission (5, 12, 48).

Although not significant, seropositivity was higher in herds that came into contact with other herds in the past year. This could be attributed to the pastoral lifestyle, which is characterized by the frequent mobility of herds. Considering the contagious nature of *Brucella* spp. sharing shared grazing areas and drinking holes makes it easier for possibly infected cows and clean herds to spread infections such as brucellosis and others (14, 62).

The current study was limited to the seroprevalence of the small ruminant brucellosis only and did not include other such as cattle and camels are susceptible to brucellosis and both species are kept in the study area but they were not part of the current study. The study also did not attempt to assess the prevalence level of the disease in humans to correlate findings in the animals. The present study did not attempt culture of *Brucella* species and therefore was not able to identify the various species and biovars of *Brucella* species circulating in sheep and goats in the study area.

5. Conclusion

The present study revealed that the seroprevalence of brucellosis in sheep and goats was found to be relatively low at both the individual animal and the herd level. However, the multivariable logistic regression analysis revealed that none of the proposed risk factors were not significantly associated with *Brucella* seropositivity at individual and herd level. In conclusion, awareness campaign among pastoral community of the seriousness of the causes, modes of transmission, symptoms, risk factors, and methods of prevention of the disease should be undertaken as soon as possible. For effective control of brucellosis that may be present in the area, an integrated approach should be promoted that takes into account the relationship between humans, animals, and the environment in the context of "One Health approach."

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal studies were approved by the Jigjiga University, College of Veterinary Medicine-Research Review Committee (Protocol No. JJU/CVM/clis/022/14). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AMH, FA, AHH, AM, and HG contributed to data collection, study design, and interpretation, manuscript draft, and writing. AMH, FA, and AHH contributed to the conception of the research idea, study design, data analysis, writing, revising and editing, the design of the study, and data interpretation. AM and HG contributed to the

reference search and manuscript writing and editing. All authors approved the submission of the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Molecular characteristics of *Staphylococcus aureus* strains isolated from subclinical mastitis of water buffaloes in Guangdong Province, China

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Intramammary infections (IMI) in animals reared for milk production can result in large economic losses and distress to the animals. *Staphylococcus aureus* is an important causative agent of IMI in dairy cows, but its prevalence in water buffaloes has not been determined. Therefore, the current study was conducted to investigate the prevalence of subclinical mastitis in water buffaloes and the antimicrobial susceptibility, virulence genes and biofilm formation abilities of *Staphylococcus aureus* isolates recovered from water buffaloes in Guangdong, China. *Staphylococcus aureus* strains were isolated from milk samples of water buffaloes with subclinical mastitis, and twofold microdilution, PCR and crystal violet staining methods were used to determine antimicrobial susceptibility, distributions of virulence and antimicrobial resistance genes and biofilm formation ability, respectively. Our results indicated that 29.44% of water buffaloes were diagnosed with subclinical mastitis, and the most prevalent pathogens were *Escherichia coli* (96.17%), coagulase-negative staphylococci (CoNS) (67.60%) and *S. aureus* (28.57%). Most *S. aureus* isolates showed resistance to bacitracin, doxycycline, penicillin, florfenicol, and tetracycline but were susceptible to ciprofloxacin, ceftizoxime, cefoquinoxime, and ofloxacin. Moreover, 63.72% of *S. aureus* isolates were positive for *tetM,* and the prevalence of *msrB*, *blaZ*, *mecA*, *fexA*, and *tetK* ranged from 21.24 to 6.19%. All *S. aureus* isolates harbored *clfB* and *icaA* genes, and the virulence genes *hla* (93.8%), *hld* (91.15%), *clfA* (90.27%), *fnbA* (86.73%), and *hlb* (83.19%), and *tsst*, *icaD*, *sec*, *see*, *fnbB*, and *sea* showed a varied prevalence ranging from 3.5 to 65.49%. All *S. aureus* isolates possessed the ability to form biofilms, and 30.09% of isolates showed strong biofilm formation abilities, while 19.47% of isolates were weak biofilm producers. Our results indicated that subclinical mastitis is prevalent in water buffaloes in Guangdong, China, and *S. aureus* is prevalent in samples from water buffaloes with subclinical mastitis. Most *S. aureus* isolates were susceptible to cephalosporins and fluoroquinolones; thus, ceftizoxime and cefoquinoxime can be used to treat subclinical mastitis in water buffaloes.

KEYWORDS

***Staphylococcus aureus*, antimicrobial resistance, virulence genes, biofilm formation, subclinical mastitis in water buffaloes**

Introduction

Water buffalo (*Bubalus bubalis*) is of importance in the milk industry and contributes to approximately 15% of milk production (1). Many parts of the world have traditionally produced buffalo milk, including Asia, Egypt, and Europe. China produces approximately 5% of global buffalo milk, and Guangdong, Guangxi and Hunan are its primary producers (2). Italy is the main producer of buffalo milk in Europe because of the popularity of buffalo mozzarella cheese (3), which retails for twice the price of bovine milk cheese (4).

Mastitis is one of the most prevalent diseases among dairy animals, causing economic losses to milk producers due to a decrease in milk quality and production, an increase in veterinary and labor costs and an increased rate of culling (5). Bacteria are the primary causative agents of mastitis, although physical trauma and mechanical injury also contribute (1). The primary bacterial causes of mastitis in both dairy cows and water buffaloes are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Escherichia coli*. *Staphylococcus aureus* is one of the primary pathogens causing mastitis in dairy cows at a level of approximately 40% in China (6).

Among antimicrobial agents, antibiotics are normally used to treat infections caused by bacteria, including intramammary infections (IMI) in domestic animals. In dairy animals, mastitis and reproductive diseases often require prolonged use of antimicrobial agents (7). Unfortunately, the widespread use of antimicrobial agents has resulted in high levels of antimicrobial resistance, leading to clinical treatment failures. Therefore, monitoring systems for antimicrobial resistance of bacterial pathogens can provide essential information for the rational use of antimicrobial agents when treating infections (8). Knowledge regarding the prevalence of mastitis and the knowledge of pathogens causing mastitis is critical in preventing the occurrence of mastitis and can provide effective measures for control and appropriate therapeutic protocols (9).

There are two groups of virulence genes in *S. aureus,* including surface-localized structural components serving as virulence factors and secreted virulence factors involved in evading host defenses and colonizing mammary glands (10). Surface-localized structural components include membrane-bound factors (fibrinogen binding protein, collagen binding protein and elastin binding protein), cell wall-bound factors (lipoteichoic acid, peptidoglycan, protein A and protease) and cell surface-associated factors (capsule). Secretory factors include toxins (staphylococcal enterotoxins, leucocidin, toxic shock syndrome toxin, and hemolysins) and enzymes (staphylokinase, coagulase, lipase, DNase, and hyaluronidase). Moreover, biofilm formation also contributes to adhesion and invasion into mammary epithelial cells and thus provides an escape from the host immune system. Enterotoxins often lead to food poisoning and include staphylococcal enterotoxins A to F and G to Q (11). Enterotoxins G to Q are more prevalent in isolates from dairy cows with mastitis than in isolates from cows without mastitis; this has implicated these virulence factors in the occurrence of mastitis (12). However, the clear mechanisms of virulence in mastitis of dairy cows need further study.

In China, the number of water buffalo farms is increasing, and thus, mastitis is occurring more frequently in these animals. Mastitis can be divided into two forms: clinical and subclinical. In clinical mastitis, clots and flakes can be observed in milk, and the quarters become swollen with severe conditions leading to the formation of lacerations, necrosis and cord formation of the teat. While no clinical signs or symptoms can be seen in subclinical mastitis, there is a reduction in milk production and deterioration of milk quality (13). Subclinical infection is 15–40 times more prevalent than clinical infection and can rapidly spread on a farm (14).

To reduce the prevalence of subclinical mastitis in water buffaloes, it is essential to have knowledge of prevalence data and understand the antimicrobial susceptibility profiles of subclinical mastitis caused by *S. aureus*. Therefore, this study was conducted to investigate the prevalence of subclinical mastitis in water buffaloes in Guangdong, China, and to determine the antimicrobial susceptibility of *S. aureus* isolates and their antimicrobial resistance and virulence determinants.

Materials and methods

Sample size

Ausvet epidemiological calculators[1] were used to calculate the sample sizes according to the method from Charan and Biswas at a 95% confidence level (15) as follows:

$$\text{Sample size} = \frac{Z^2_{1-\frac{\alpha}{2}} P(1-p)}{d^2}$$

where $z_{1-\alpha/2}$ equals 2.58 with a 1% type error ($p<0.01$). The expected proportion of water buffaloes in Guangdong Province is 0.05, and the expected precision (d) is 0.01.

A total of 3,900 samples from 975 water buffaloes were included in this study from the following regions: Qingyuan ($n=884$), Guangzhou ($n=1,076$), Jiangmen ($n=992$) and Zhaoqing ($n=948$). The average sample number per farm was 342.9 (range 174 to 584).

Sample collection

The milking of animals on all farms was performed twice a day, and the sample collection procedure consisted of fore-stripping (3–5 squirts of milk) followed by teat disinfection with 0.25% iodine solution and drying with a clean towel. The milking clusters were then attached and removed automatically when finished, followed by postmilking teat disinfection with 0.5% iodine. Duplicate milk samples for each quarter were aseptically collected according to standard protocols of the National Mastitis Council (16). Briefly, milk samples (3 mL) were collected from all quarters of each animal after the first 3 streams of milk were discarded and placed in an ice box and transferred to the laboratory within 6 h. Presumptive evidence of subclinical mastitis (17) was determined using a commercial California Mastitis Test kit (CMT) (ImmuCell, Portland, ME, United States) following the recommendations of the manufacturer. Briefly, 2 mL of milk sample was mixed with an equal amount of CMT solution in the paddle and stirred for 30 s. Thickening indicated elevated somatic cell counts (SCC), and these samples were then used for bacterial isolation and identification.

1 https://ausvet.com.au/

Bacterial isolation

Identification of *S. aureus* from milk samples was carried out as previously described (18). Briefly, a 0.1 mL milk sample was inoculated into 3 mL tryptic soy broth (TSB; AoBox, Beijing, China) containing 10% NaCl and incubated at 37°C for 24 h. A loopful of enrichment broth culture was streaked onto a Baird-Parker agar plate (AoBox) and incubated at 37°C for 24 h. The suspected *S. aureus* colonies had a black and shiny appearance with a thin white border surrounded by a light area. The suspected colonies were streaked onto chromogenic *S. aureus* agar plates (CHROMagar, Paris, France). At least 3 positive colonies per sample were then confirmed as coagulase-positive *S. aureus* using commercial API STAPH test strips (bioMérieux, Marcy-l'Ètoile, France). *Staphylococcus aureus* isolates were stored at −80°C in cryogenic vials (Biologix, Shandong, China) containing 1 mL TSB and 30% glycerin. Coagulase-negative staphylococci were isolated as previously described (19). In brief, milk samples were cultured on blood agar plates (AoBox) at 37°C for 48 h. Typical colonies were selected and identified using classical biochemical methods, including Gram staining and oxidase, catalase and DNase tests, and the ability to coagulate rabbit plasma using commercial kits (Sigma Chemical, Shanghai, China). If the suspected strains failed biochemical identification, molecular identification using PCR amplification and sequencing of the *sodA* gene was performed as previously described (19).

Escherichia coli isolation utilized a 0.1 mL milk sample inoculated in 3 mL Mueller-Hinton broth (MHB; Aobox), which was incubated at 37°C for 24 h. Samples were then streaked onto MacConkey Agar plates, and plates were kept at 37°C for 24 h. Presumptive identification of *E. coli* were pink colonies that were then subjected to matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using a Microflex LT instrument (Bruker Daltonics, Bremen, Germany).

Streptococcus agalactiae and *S. dysgalactiae* were identified as previously described (20) using EN medium, and positive isolates were then transferred to Columbia Blood Agar Base Medium containing 5% sheep blood (Hope Bio-Technology, Qingdao, China) and incubated at 37°C for 24 h. Colonies with typical *Streptococcus* morphologies were then subjected to catalase and 6.5% NaCl tests. Isolates were further identified as *S. agalactiae* and *S. dysgalactiae* according to reactions using the sodium hippurate test, esculin hydrate, and CAMP tests.

Antimicrobial susceptibility testing

Antimicrobial resistance phenotypes of *S. aureus* isolates were determined using the microdilution method in MH broth according to the Clinical Laboratory and Standards Institute guidelines (CLSI) (21). A loopful of each *S. aureus* isolate preserved in glycerinated TSB was streaked on a Baird-Parker agar plate and incubated at 37°C for 24 h. The colonies were inoculated in MH broth, and the cultures were diluted in sterile normal saline and adjusted to a turbidity of 0.5 McFarland standard (10^5–10^6 colony-forming units (CFU)/mL). The suspension was then swabbed on Muller-Hinton agar plates and incubated at 37°C for 24 h as previously described. *Staphylococcus aureus* ATCC 25923 was used as the reference strain. Each experiment was repeated at least three times. Breakpoints for different antimicrobial agents were based on CLSI guidelines (21).

Antimicrobial agents included ceftiofur (CTF) (0.02–16 μg/mL), cefoquinoxime (CFQ, 0.03–32 μg/mL), ceftizoxime (CFT, 0.03–32 μg/mL), cefoxitin (CFX, 0.03–32 μg/mL), florfenicol (FLO, 0.125–128 μg/mL), ciprofloxacin (CIP, 0.03–32 μg/mL), enrofloxacin (ENO, 0.03–32 μg/mL), ofloxaxin (OFX, 0.06–64 μg/mL), erythromycin (ERY, 0.125–128 μg/mL), azithromycin (ATM, 0.125–128 μg/mL), gentamycin (GEN, 0.125–128 μg/mL), penicillin (PEN, 0.03–32 μg/mL), ampicillin (AMP, 0.125–128 μg/mL), tetracycline (TET, 0.125–128 μg/mL), doxycycline (DXC, 0.03–32 μg/mL) and bacitracin (BTC, 4–1024 μg/mL).

Antimicrobial resistance and virulence gene detection

Strains used for testing were taken from frozen stocks, cultures were streaked onto TSA plates containing 5% sterile defibrinated sheep blood, and the plates were incubated at 37°C for 24 h. Single colonies were inoculated into 3 mL TSB and cultured with shaking at 37°C for 24 h. The cultures were centrifuged at 3000 × *g*, and cell pellets were suspended in phosphate buffered saline (PBS, pH 7.4; Solarbio, Beijing, China) containing 20 mg/mL lysostaphin (Meilunbio, Dalian, China). The mixture was kept at 37°C for 30 min, genomic DNA was extracted using a TIANamp bacterial DNA extraction kit (TianGen, Beijing, China), and DNA quality was evaluated by UV spectroscopy with a NanoDrop-2000 instrument (Thermo Fisher, Shanghai, China). The extracted DNA was diluted to 50 mg/L in sterile deionized water for PCR assays (see below).

Antimicrobial resistance genes (ARGs) were detected using multiplex PCR as previously described (22). Briefly, PCRs included gene-specific primers for the following ARG groups: penicillin (*blaZ*), macrolide (*msrA* and *msrB*), erythromycin (*ermA* and *ermC*), streptogramin acetyltransferase genes (*vatA*, *vatB*, and *vatC*), aminoglycoside (*aacA-D*), tetracycline (*tetK* and *tetM*), lincosamide (*linA*), methicillin (*mecA*), florfenicol (*fexA*), oxazolidine ketone (*cfr* and *optrA*) and vancomycin (*vgaA* and *vgaC*).

Virulence genes (*hla*, *hlb*, *hld*, *sea*, *seb*, *sec*, *sed*, *see*, *tst*, and *lukF*), biofilm-related genes (*bap*, *icaA*, and *icaD*) and adhesion-related genes (*fnbA*, *fnbB*, *clfA*, and *clfB*) were detected using PCR as previously described (23). The primers were provided by Sangon Biotech (Shanghai, China), and water rather than DNA template was added as a contamination control. DNA from isolates that harbored virulence genes or ARGs was used as a positive control. These were included in all PCRs. Gene amplifications were performed using a commercial PCR instrument (Bio-Rad, Hercules, CA, United States) as previously described (24). Briefly, the PCR mixture contained DNA (1 μL), 0.2 μL of each primer, Prime STAR Max DNA polymerase (12.5 μL), and ddH_2O (11.1 μL). The PCR cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, annealing at appropriate temperatures for 30 s (Supplementary Table S1) and extension at 72°C for 1 min.

Biofilm formation

Biofilm formation was determined in 96-well microtiter plate assays using minimal medium M9 (Sigma Chemical) as previously described (25). Briefly, the overnight cultures in TSB were diluted

TABLE 1 Antimicrobial susceptibility of *S. aureus* isolates from subclinical mastitis of buffaloes.

Antimicrobial agents	MICs (μg/mL)			Break point	Resistance	Mediate	Susceptibility
	MIC50	MIC90	Range				
Penicillin	4	32	0.03– ≥ 128	≤0.12, –, ≥0.25	84.96% (96/113)		15.04% (17/113)
Ampicillin	0.125	16	0.125– ≥ 128	≤0.25, –, ≥ 0.5	55.75% (63/113)		54.25% (50/113)
Cefoquinoxime	0.06	16	0.06–32	≤2, 4, ≥ 8	18.58% (21/113)	12.39% (14/113)	69.03% (78/113)
Ceftizoxime	0.12	8	0.12–32	≤2, 4, ≥ 8	15.04% (17/113)	10.62% (12/113)	74.34% (84/113)
Ceftiofur	4	8	0.125– ≥ 128	≤2, 4, ≥ 8	27.43% (31/113)	36.28% (41/113)	36.28% (41/113)
Cefoxitin	0.012	16	0.006–32	≤4, –, ≥8	10.62% (12/113)	0	89.38% (101/113)
Chloramphenicol	16	≥128	0.5– ≥ 128	≤4, 8, ≥ 16	81.42% (92/113)	10.62% (12/113)	7.96% (9/113)
Ciprofloxacin	0.5	4	0.125–64	≤1, 2, ≥ 4	7.08% (8/113)	15.04% (17/113)	77.88% (88/113)
Ofloxacin	0.5	32	0.06–64	≤1, 2–4, ≥ 8	16.81% (19/113)	19.47% (22/113)	63.72% (72/113)
Enrofloxacin	1	32	0.06– ≥ 128	≤0.5, 1–2, ≥ 4	37.17% (42/113)	20.35% (23/113)	42.48% (48/113)
Erythromycin	≥128	≥128	0.125– ≥ 128	≤0.5, 1–4, ≥ 8	74.34% (84/113)	7.08% (8/113)	18.58% (21/113)
Azithromycin	4	≥128	0.06– ≥ 128	≤2, 4, ≥ 8	32.74% (37/113)	19.47% (22/113)	47.79% (54/113)
Gentamicin	1	32	0.125– ≥ 128	≤4, 8, ≥ 16	21.24% (24/113)	15.04% (17/113)	63.72% (72/113)
Tetracycline	8	≥128	0.125– ≥ 128	≤0.25, 0.5, ≥ 1	82.3% (93/113)	2.65% (3/113)	15.04% (17/113)
Doxycycline	8	32	0.125–32	≤0.12, 0.25, ≥ 0.5	84.07% (95/113)	3.54% (4/113)	12.39% (14/113)
Bacitracin	256	512	4– ≥ 1,024	≤64, 128, ≥ 256	90.27% (102/113)	3.54% (4/113)	6.19% (7/113)

1:100, and 200 μL was transferred into each well of the microtiter plate that was incubated at 37°C for 72 h. Each well was washed with 200 μL PBS after the supernatant was discarded and fixed with 200 μL methanol for 20 min and washed again with PBS 3 × and then stained with 0.4% crystal violet (Meilunbio, Dalian, China) for 15 min. The biofilms were then dissolved in 200 μL 33% (w/v) acetic acid for 30 min. The biofilm formation was measured at 590 nm optical density ($OD_{590\ nm}$) in a Bio-Rad plate reader (Shanghai, China). The strong biofilm-forming strain *Salmonella enterica* Typhimurium ATCC 14028 was used as the positive control, and sterile TSB was used as the negative control for the biofilm formation assay (26). All assays were performed in triplicate. The $OD_{590\ nm}$ value of 0.6 was applied as the cutoff point to distinguish between biofilm producers and nonbiofilm producers (10). Biofilm formation was classified as strong +++ ($OD_{590\ nm} > 1.8$), moderate ++ ($1.8 > OD_{590\ nm} > 1.2$), weak + ($1.2 > OD_{590\ nm} > 0.6$), and negative – ($OD_{590\ nm} < 0.6$).

Statistical analysis

Pearson correlation analysis was applied to differences in antimicrobial resistance rates in correlation to antimicrobial resistance genes harbored by *S. aureus* isolates. *T* tests were used to analyze the significance of biofilm formation between *S. aureus* isolates. All analyses were carried out using Prism 8 (GraphPad, Boston, MA, United States).

Results

Prevalence of subclinical mastitis in water buffaloes

Our screening of 975 water buffaloes indicated that 287 (29.44%) were considered to have subclinical mastitis according to the CMT tests. Strongly positive (+++) results were observed in 53.31% (153/287) of the cases, while mild and moderate intensity results occurred in 26.13% (75/287) and 20.56% (59/287) of the cases, respectively. *Escherichia coli* (276/287) was the most common bacteria isolated from these positive samples, followed by coagulase-negative staphylococci (CNS) (194/287). *Staphylococcus aureus* (113/287), *S. agalactiae* (82/287) and *S. dysgalactiae* (41/287).

Antimicrobial resistance phenotype

Resistance to bacitracin, doxycycline, penicillin, florfenicol and tetracycline was observed in 90.27, 84.07, 84.96, 81.42 and 82.3% of the examined *S. aureus* isolates, respectively (Table 1). A lower prevalence of resistance was noted for ciprofloxacin (7.08%), ceftizoxime (15.04%), cefoquinoxime (18.58%) and ofloxacin (16.81%). Among *S. aureus* isolates, only 12 (10.62%) were resistant to cefoxitin, and these were classified as phenotypic methicillin-resistant *S. aureus* (MRSA).

Distribution of antimicrobial resistance genes among *Staphylococcus aureus* isolates

The ARGs possessed by *S. aureus* isolates included *tetM*, *ermC*, *vatC*, and *aacA-D* in 63.72% (72/113), 37.17% (42/113), 32.74% (37/113), and 27.43% (31/113), respectively. Moreover, the prevalence of *msrB*, *blaZ*, *mecA*, *fexA*, and *tetK* was 21.24, 19.47, 16.81, 15.04, and 6.19%, respectively (Figure 1). Interestingly, ARGs for macrolide resistance *msrA*, erythromycin resistance *ermA*, streptogramin resistance *vatA* and *vatB*, oxazolidinone resistance *cfr* and *optrA* and vancomycin resistance *vgaA* and *vgaC* were not detected. These results indicated a lack of a correlation between resistance phenotypes and ARG distributions among the *S. aureus* isolates.

Prevalence of virulence-associated genes

The virulence-associated genes we detected in this study were distributed with varying frequencies among *S. aureus* isolates ($n = 113$) (Figure 2). In particular, *clfB* and *icaA* were present in all *S. aureus* isolates, and nearly all harbored *hla* (93.8%), *hld* (91.15%), *clfA* (90.27%), *fnbA* (86.73%) and *hlb* (83.19%). In contrast, a lower prevalence was found for *tsst* (27.43%), *icaD* (19.47%), *sec* (15.93%), *see* (9.73%), *fnbB* (65.49%) and *sea* (3.54%). The virulence genes *seb*, *sed*, *bap*, and *lukF* were not detected in any isolates.

Biofilm formation ability

All our milk samples produced isolates able to form biofilms. The rates of strong, moderate and weak biofilm producers were 30.09, 50.44, and 19.47%, respectively. In particular, most Qingyuan isolates (70.83%, 17/24) were strong biofilm producers, while 20.83% displayed moderate phenotypes. In contrast, only 9.76% (4/41) of the Guangzhou isolates were strong biofilm producers, and 65.85% (27/41) were moderate producers. Biofilm formation in *S. aureus* isolates from Guangzhou, Qingyuan, Jiangmen and Zhaoqing was 0.69 ± 0.24, 1.16 ± 0.35, 1.05 ± 0.25, and 1.03 ± 0.28, respectively. The biofilm formation of isolates from Guangzhou was significantly lower ($p < 0.01$) than that of isolates from other areas (Figure 3; Table 2).

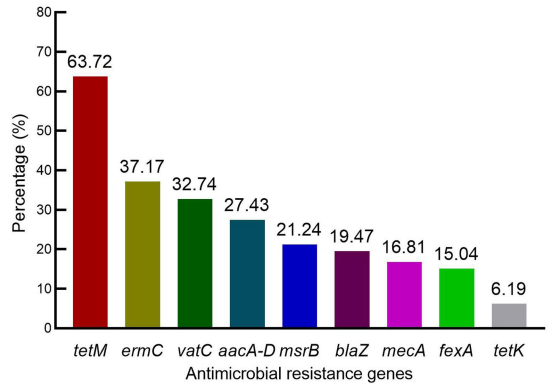

FIGURE 1
Distribution of antimicrobial resistance genes among *S. aureus* isolates.

Discussion

Mastitis is a disease that is globally prevalent in dairy animals (1), and water buffaloes are generally less susceptible to this infection in comparison with dairy cows because of strong muscles at the opening of the teat canal (27). In the current study, 29.44% of water buffaloes were diagnosed with subclinical mastitis, which was consistent with a previous report for these animals (28). Previous studies have indicated that the prevalence of subclinical mastitis in water buffaloes ranges from 6.0 to 87% (27). It seems that factors such as animal age, stage of lactation, management style and farm environment may have contributed to these variations (13). To our knowledge, few studies have been carried out to investigate the bacteriology of subclinical mastitis in water buffaloes. In this study, we found that CoNS, *E. coli* and *S. aureus* were the dominant bacterial pathogens and that *S. agalactiae* and *S. dysgalactiae* were present to a lesser degree. *Escherichia coli* infections often lead to severe systemic clinical symptoms, and this was the most prevalent pathogen in our study. Similarly, *E. coli* was the most prevalent pathogen in subclinical mastitis infections in a Nepal water buffalo study (28), while *Streptococcus* (39.2%) was the most prevalent pathogen in mastitis dairy cows, and only 8.4% were present as *E. coli* (29). Therefore, it seems that other factors (see above) may influence the occurrence of mastitis. Moreover, *S. aureus* (61%) was the dominant pathogen in cattle from Jammu and Kashmir

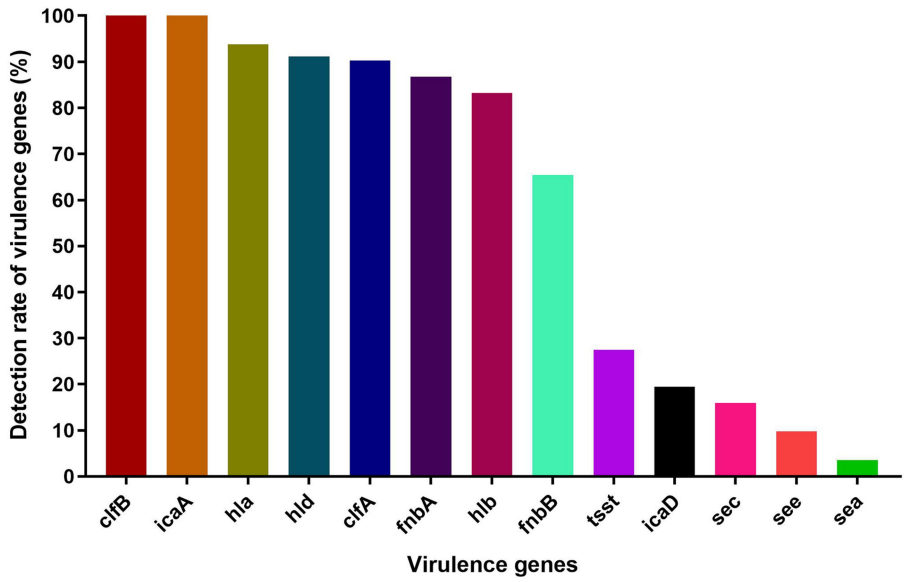

FIGURE 2
The detection rate of virulence genes among *S. aureus* isolates.

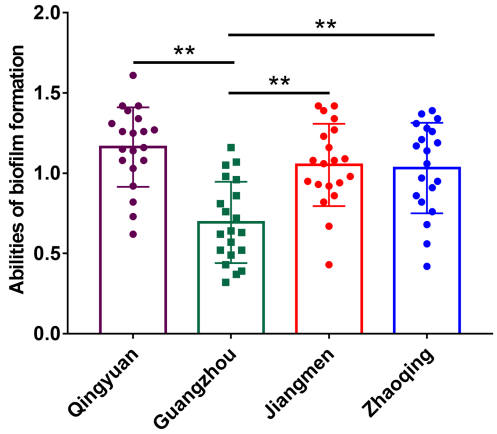

FIGURE 3
Biofilm formation of *S. aureus* isolates in different areas of Guangdong. Note: There were no significant difference between *S. aureus* isolates from Qingyuan, Jiangmen, and Zhaoqing. But *S. aureus* isolates from Guangzhou were significantly lower ($p < 0.01$) in comparison with isolates from Qingyuan, Jiangmen, and Zhaoqing. ** means $p < 0.01$.

TABLE 2 Biofilm formation ability of *S. aureus* isolates from water buffaloes.

Areas	Strong biofilm formation	Mediate biofilm formation	Weak biofilm formation
Qingyuan	70.83% (17/24)	16.67% (4/24)	12.5% (3/24)
Guangzhou	9.76% (4/41)	65.85% (27/41)	24.39% (10/41)
Jiangmen	18.52% (5/27)	51.85% (14/27)	25.93% (7/27)
Zhaoqing	38.1% (8/21)	42.86% (9/21)	16.67% (4/21)
Average	30.09% (34/113)	50.44% (57/113)	19.47% (22/113)

with mastitis, and *E. coli* (13%), CoNS (13.04%), *Streptococcus uberis* (4.35%) and *S. dysgalactiae* (8.69%) were also isolated from samples (30). Similar results were reported by other researchers (27); the reasons for this might be topographical and management conditions and the difference between dairy cows and water buffaloes.

Antimicrobial susceptibility can provide important information in choosing antimicrobial agents when treating infections. In this study, *S. aureus* isolates showed high resistance to penicillin, florfenicol, erythromycin, tetracycline, doxycycline and bacitracin. Similar results were observed in *S. aureus* isolates from dairy cows with mastitis in northern China (31). However, other researchers reported lower rates of antimicrobial resistance in *S. aureus* isolates in Pakistan (13). Cephalosporins are important antimicrobial agents, and *S. aureus* isolates resistant to ceftiofur have been reported (23). Similarly, antimicrobial resistance to cephalosporins, including cefoquinoxime, ceftizoxime, and ceftiofur, was detected in our study. β-lactams, fluoroquinolones, and aminoglycosides are commonly used to treat dairy mastitis (31), and this most likely contributed to the high levels of resistance we found to these agents. Saini and his colleagues also found that the herd level of antimicrobial agents used when treating mastitis in bovines was positively correlated with antimicrobial resistance among isolates from mastitic animals (32). Unfortunately, the use of antimicrobial agents in these farms was not documented in our study, so we cannot directly correlate the use of antimicrobial agents and antimicrobial resistance. Moreover, we detected antimicrobial resistance genes among isolates, although we found no significant correlation between phenotype and genotype. For example, only 22.92% (22/96) of isolates resistant to penicillin carried the *blaZ* gene, similar to previous findings (33, 34). These inconsistencies indicated that the presence of a particular ARG was not an indicator of phenotypic resistance, and this can be influenced by numerous genetic and environmental conditions (35).

MRSA is a global health concern since it is not only resistant to β-lactams but also nonsusceptible to other commonly used antimicrobial agents (36). In the Philippines and Pakistan, the MRSA prevalence was 25.81 and 19.6%, respectively, in water buffaloes with mastitis (13, 37), while a much lower rate (2.2%) of MRSA was detected in water buffaloes with mastitis in Iran (38). Several factors, such as age, feeding status, body conditions, and hand or machine hygiene on the farm, may contribute to this phenomenon. Several technologies, such as nanoparticles and antibiotics combined with plant extracts or microparticles, are widely used in food, veterinary and animal science. For example, a report indicated that antibiotics coupled with zinc oxide nanoparticles can significantly increase the zone of inhibition; similarly, amoxicillin showed the highest increase in inhibitory effects against MRSA when combined with *Calotropis procera* extract (39). These technologies are believed to be promising methods for treating infections caused by MRSA.

Biofilms can increase the resistance of *S. aureus* to antimicrobial agents and are responsible for persistent infections (40). Biofilms are composed of multiple layers of bacteria, which prevents the permeability of antimicrobial agents and thus increases tolerance. In our study, we investigated the biofilm formation ability of *S. aureus* isolates grouped by area. Interestingly, *S. aureus* isolates from Guangzhou showed significantly lower levels of biofilm formation in comparison with isolates from other areas ($p < 0.01$). However, the antimicrobial resistance of *S. aureus* isolates did not differ by region (data not shown). Similarly, a previous study indicated that the biofilm formation ability of ST7 and ST188 strains was much higher than that of other lineages even though their phenotypic antimicrobial resistance was comparable with that of other lineages (41). These data indicated that gene mutations, horizontal gene transfer and modifications of antibiotic molecules are the primary modes of antimicrobial resistance in *S. aureus* isolates from water buffaloes and that biofilm formation plays only a secondary role (42).

Virulence genes contribute to the pathogenesis of *S. aureus* infections. Adhesion is the first step for *S. aureus* to invade host cells and immune responses (43) and involves *clfA*, *clfB*, *fnbA*, and *fnbB*. In our study, all isolates carried *clfB,* and most isolates carried *clfA*, *fnbA*, and *fnbB*. These results were similar to previous reports where the *clfB* gene was detected in all isolates from bovine mastitis samples, and *fnbA* and *clfB* were comparable with the levels we found (44, 45). In contrast, much lower detection levels were reported for *fnbB* in *S. aureus* isolates from Algeria and Australia (43, 46).

Hemolysins are also involved in invasion and the host immune response (44, 46). In our study, over 80% of our total isolates carried *hla*, *hlb*, and *hld,* consistent with previous reports (41, 44). Toxic shock syndrome toxin, a superantigen encoded by the *tsst* gene, can lead to toxic shock syndrome in humans (47). and the *tsst* prevalence in *S. aureus* isolates ranged from 2.1 to 40.0% (10, 44) and was 25% in our study. It is therefore important to monitor the epidemiology of such super antigenic toxin genes to protect public health from this threat.

Conclusion

In conclusion, subclinical mastitis was prevalent among water buffaloes in Guangdong, China, and *S. aureus* was identified as a significant pathogen associated with subclinical mastitis of water buffaloes. The majority of *S. aureus* isolates exhibited resistance against bacitracin, doxycycline, penicillin, florfenicol, and tetracycline while maintaining susceptibility to other antimicrobial agents, including ciprofloxacin, ceftizoxime, cefoquinoxime, and ofloxacin. Furthermore, the *S. aureus* isolates harbored various virulence genes, such as *hla*, *hld*, *clfA*, *fnbA*, and *hlb*. Notably, all *S. aureus* isolates showed the ability to form biofilms, with nearly one-third of the isolates possessing strong biofilm formation abilities. Given these findings, antibiotics should be cautiously used when treating subclinical mastitis in water buffaloes within this region. Additionally, the impact of biofilm formation on the transmission of antibiotic resistance must be investigated in further studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal studies were approved by the Ethical Committee of Foshan University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

DZ, NZ, and HY: conceptualization and writing – review and editing. XL and XS: methodology and data curation. XF and QL: analysis. DZ: writing original draft preparation and funding acquisition. HY: supervision and project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1177302/full#supplementary-material

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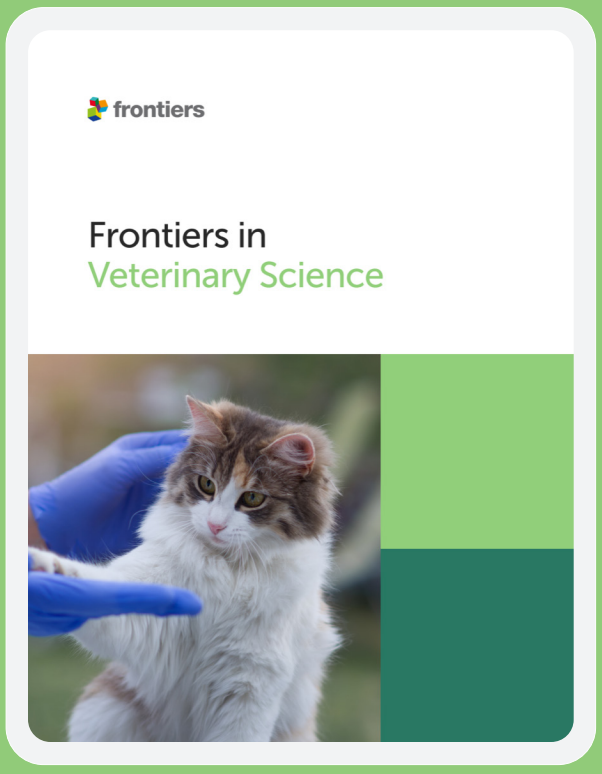

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