PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 46:e07754, 2026
DOI: 10.1590/1678-5150-PVB-7754

Original Article
Livestock Diseases

Mass spectrometry-based identification reveals the complexity of microorganisms involved in umbilical infections in lambs clinically scored[1]

Thaís S. Bello[2]*, Fábio V.R. Portilho[2], Lorrayne S.M. Motta[3], Rodrigo G. Motta[3], Geraldo Nardi Júnior[4], Fábio S. Possebon[2], Rodolfo J.C. Souto[5], José A.B. Afonso[5], Patrik Jr. L. Paz[2], Márcio G. Ribeiro[2]

ABSTRACT.- Bello TS, Portilho FVR, Motta LSM, Motta RG, Nardi Júnior G, Possebon FS, Souto RJC, Afonso JAB, Paz PJrL, Ribeiro MG. **Mass spectrometry-based identification reveals the complexity of microorganisms involved in umbilical infections in lambs clinically scored.** *Pesquisa Veterinária Brasileira 46:e07754, 2026.* Departamento de Produção Animal e Medicina Veterinária Preventiva, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Walter Maurício Correa s/n, Botucatu, SP 18618-681, Brazil. E-mail: thais.bello@unesp.br

We investigated the microbiological etiology of umbilical infections in clinically scored lambs. A total of 128 lambs showing signs of umbilical infection and 41 showing no umbilical signs were sampled. All microorganisms were identified by mass spectrometry (MALDI-TOF MS), and the *in vitro* antimicrobial susceptibility/resistance patterns of bacteria were assessed. Among the diseased animals and those without umbilical signs, 214 and 116 species of microorganisms (bacteria and yeasts) were identified, respectively. *Escherichia coli* (11.21%) was the most prevalent microorganism and showed a significant association ($p = 0.0039$) with moderate (score 2) and severe (score 3) scores for umbilical infection. In lambs without clinical signs of umbilical infection, a predominance of *Desemzia incerta* was observed. Diseased lambs that exhibited clinical complications showed a high mortality rate (58%). Additionally, a significant association ($p < 0.001$) was observed between moderate and severe scores and poor prognosis. Bacterial multidrug resistance was observed in 20% (36/182) of isolates. To our knowledge, some bacteria were identified for the first time as primary agents of umbilical infections in lambs, and clinical scoring was applied for the first time in newborn lambs with omphalopathies. Our findings reveal a high complexity of microorganisms in umbilical infections in lambs, identified by mass spectrometry, with a predominance of *E. coli* in cases of moderate to severe severity scores, and an association between clinical complications and high mortality.

INDEX TERMS: Infectious omphalopathies, sheep, etiology, multidrug-resistant bacteria, MALDI-TOF MS.

RESUMO.- [**Espectrometria de massas revela complexidade de micro-organismos isolados em infecções umbilicais em cordeiros com avaliação dos escores de gravidade clínica.**] A etiologia microbiana das infecções umbilicais em cordeiros, com avaliação dos escores de gravidade clínica, foi investigada em 128 animais com sinais de infecção umbilical e 41 aparentemente sadios. Todos os microrganismos foram identificados por espectrometria de massas (MALDI-TOF MS), assim como foi investigado o perfil *in vitro* de sensibilidade/resistência aos antimicrobianos dos isolados bacterianos. Nos animais com e sem infecção umbilical foram identificadas 214 e 116 espécies de microrganismos (bactérias e leveduras), respectivamente. *Escherichia coli* (11,21%) foi o microrganismo

[1] Received on September 25, 2025.
Accepted for publication on December 1, 2025.

[2] Departamento de Produção Animal e Medicina Veterinária Preventiva (DPAMVP), Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista "Júlio de Mesquita Filho" (Unesp), Rua Prof. Walter Maurício Corrêa s/n, Botucatu, SP 18618-681, Brazil. *Corresponding author: thais.bello@unesp.br

[3] Departamento de Medicina Veterinária , Universidade Estadual de Maringá (UEM), Umuarama, PR 87507-190, Brazil.

[4] Produção Animal e Agronegócio, Faculdade de Tecnologia do Estado de São Paulo (FATEC), Botucatu, SP 18606-851, Brazil.

[5] Clínica de Bovinos , Universidade Federal Rural de Pernambuco (UFRPE), Garanhuns, PE 55292-270, Brazil.

mais prevalente e mostrou associação significante (p = 0,0039) com escores moderados (escore 2) e graves (escore 3) para infecção umbilical. Em cordeiros sem sinais clínicos de infecção umbilical observou-se o predomínio de *Desemzia incerta*. Cordeiros com infecção umbilical que apresentaram complicações clínicas apresentaram elevada mortalidade (58%). Foi observada ainda associação significante (p < 0,001) entre escores de gravidade clínica moderado e grave e prognóstico desfavorável. A multirresistência bacteriana foi observada em 20% (36/182) dos isolados. Na literatura consultada, certas bactérias foram identificadas pela primeira vez como agentes primários de infecções umbilicais em cordeiros, bem como escores de gravidade clínica foram adotados pioneiramente em cordeiros neonatos com onfalopatias. O presente estudo revelou alta complexidade de microrganismos nas infecções umbilicais em cordeiros indetificados por espectrometria de massas, com predomínio de *E. coli* nos casos moderados e graves e alta taxa de mortalidade nas infecções umbilicais com complicações clínicas.

TERMOS DE INDEXAÇÃO: Onfalopatias infecciosas, ovelha, etiologia, bactérias multidroga resistentes, MALDI-TOF MS.

INTRODUCTION

Infectious diseases in domestic ruminants are the main causes of morbidity, mortality, and losses for farmers during the first three months of life (Tora et al. 2021, Ismail & Muhaffel 2022, Mee 2023). Umbilical infections in lambs have been associated with severe complications of bacterial dissemination (e.g., arthritis, abscesses in organs, pneumonia, and sepsis), and a high mortality rate estimated between 6% and 31.3% (Holmøy et al. 2012, Sato et al. 2021, Smistad et al. 2021, Van Camp et al. 2022).

The inadequate transfer of passive immunity by colostrum, improper umbilical antisepsis postpartum, and high organic matter (feces) in the environment are considered risk factors for mortality in lambs related to infectious diseases, including umbilical infections (Binns et al. 2002, Giadinis et al. 2009, Wieland et al. 2017, Barry et al. 2022, Tüfekci & Sejian 2023, Farooq et al. 2024), particularly in tropical areas (Hordofa et al. 2021).

Umbilical infections in domestic ruminants have a highly complex etiology, with bacteria being the predominant agent, particularly enterobacteria (e.g., *Escherichia coli* and *Klebsiella pneumoniae*), staphylococci, streptococci, and actinomycetes species (e.g., *Trueperella pyogenes*) (Rutherford et al. 2014, Ribeiro et al. 2015, Martins et al. 2023). The microorganisms infect the umbilical cord through direct contact with the environment via dirt, organic matter, soil, feces, and common-use utensils (Windeyer et al. 2014, Van Camp et al. 2022), or through the microbiota of the umbilical region (Quinn et al. 2011, Constable et al. 2017).

Edema, congestion, and discomfort upon palpation, with or without purulent discharge, are the most common clinical signs of umbilical infections in domestic ruminants (Steerforth & Van Winden 2018, Van Camp et al. 2022). In calves, umbilical infection severity has been scored as follows: 1 (mild), 2 (moderate), and 3 (severe) (Steerforth & Van Winden 2018). Nonetheless, a lack of severity scores identification has been reported for umbilical infections in lambs.

The routine diagnosis of umbilical infections in lambs is based on clinical and epidemiological findings, microbiological culture, and *in vitro* antimicrobial susceptibility patterns (Underwood et al. 2015, Steerforth & Van Winden 2018). However, the diagnosis of the microorganisms identified in umbilical infections in lambs at the species level has usually been based on conventional phenotypic tests (Rutherford et al. 2014, Ribeiro et al. 2015). Also, a lack of molecular identification of etiological agents has been observed.

The treatment approaches for umbilical infections in lambs have been based on broad-spectrum antimicrobials, particularly beta-lactam derivatives, fluoroquinolones, aminoglycosides, sulfonamides, and tetracyclines, including drugs available for intravenous administration (Scott et al. 2011, Underwood et al. 2015). Additionally, the use of nonsteroidal anti-inflammatory drugs (e.g., meloxicam) and fluid therapy is recommended in cases of systemic complications related to sepsis (Wieland et al. 2017).

Considering this scenario, we investigated the etiology of umbilical infection in lambs diagnosed by mass spectrometry, the clinical severity scores of umbilical infections, the occurrence of *in vitro* multidrug-resistant isolates, and the outcomes of cases with clinical complications.

MATERIALS AND METHODS

Ethical approval. This study was carried out following the Ethics Committee on Animal Use (CEUA) guidelines of the "Faculdade de Medicina Veterinária e Zootecnia" (School of Veterinary Medicine and Animal Sciences – FMVZ), "Universidade Estadual Paulista 'Júlio de Mesquita Filho'" (Unesp), Botucatu/SP, Brazil (protocol number 0260/2022).

Animals and sampling. A sampling of 128 lambs of various breeds (e.g., Dorper, Texel, Santa Inês, and crossbred) with umbilical infections was collected from the states of São Paulo (Southeast), Paraná (South), and Pernambuco (Northeast), Brazil. Additionally, 41 lambs without apparent clinical signs of umbilical infection were also conveniently collected in the states of São Paulo and Paraná, Brazil. The sampled lambs with signs of umbilical infection were, on average, 6.5 (1–30) days old (1–7 days, 84/122, 69%; 8–30 days, 38/122, 31%). Of these, 54% (68/126) were female, and 46% (58/126) were male. Among those lambs without apparent clinical signs of umbilical infection, 54% (22/41) were female, and 46% (19/41) were male.

The sample size was calculated to estimate the prevalence associated with a margin of error. The prevalence (4%) was based on a previous study (Gökçe & Erdoğan 2009) and was associated with a 95% confidence interval and a margin of error of ± 5%. Based on this, at least 60 lambs with umbilical infections should be included in the study. Lambs without clinical signs of umbilical disease were collected by convenience sampling from the same farms where diseased lambs were sampled.

Farms and flocks were eligible for lamb sampling if they met the following criteria: 1) maternity pens, 2) absence of antimicrobial treatment seven days before umbilical sampling, 3) animals no older than 60 days of life with an umbilical infection, and 4) lambs within one day of age without apparent clinical signs of umbilical infection.

All the animals included in this study were bred under similar conditions of nutrition, management, sanitary procedures (including vaccines and the use of anthelmintic drugs), and facilities on farms.

The lambs were manually restrained, and samples were collected using disposable gloves and sterile swabs after rigorous antisepsis (70% alcohol and 2% iodine) of the external region of the umbilical cord. The swabs were fully inserted into the navel without touching the external region, immediately placed into Stuart transport media

(Absorve™, Hangzhou, China), and kept refrigerated (4–8 °C) for further microbiological procedures. All umbilical samples were collected by trained veterinarians, who could also identify the clinical severity scores.

Clinical severity scores and complications of umbilical infections. Clinical severity scores (CSS) of umbilical infections used in the sampled lambs were adapted from Steerforth & Van Winden (2018) for calves. Score 0 (normal): umbilical structures involuting, absence of pain, swelling, or any other signs of infection or inflammation, lambs without apparent clinical umbilical infection. Score 1 (mild umbilical infection): pain on palpation, higher local temperature, hardening and/or swelling of the umbilical cord (thickness greater than a pencil or 6 mm), absence of other clinical signs. Score 2 (moderate umbilical infection): presence of pus and increased thickness of the umbilical cord (> 6 mm). Score 3 (severe umbilical infection): clinical signs found in score 2 plus increased rectal temperature (40 °C), systemic signs (fever, lethargy, apathy, tachycardia, respiratory distress, lack of appetite/anorexia), or the presence of a hernia.

Data on probable complications of umbilical infections (i.e., arthritis, pneumonia, sepsis) and their outcomes (death or recovery) were also assessed up to six months after umbilical sampling.

Bacteriological and fungal cultures. Umbilical samples were cultured under aerobic conditions in bovine blood agar (5%) (Oxoid®, São Paulo, Brazil) and MacConkey agar (Oxoid®, São Paulo, Brazil) and incubated for 72 hours at 37 °C. The samples were also cultured in Sabouraud-dextrose agar media supplemented with 0.5% chloramphenicol (Oxoid®, São Paulo, Brazil), incubated at 37 °C, and evaluated for 72 hours. Simultaneously, the samples were cultured under anaerobic conditions in bovine blood agar (5%) (Oxoid®, São Paulo, Brazil) and in brain heart infusion (BHI) (Oxoid®, São Paulo, Brazil) and incubated for 120 hours at 37 °C. The microorganisms were preliminarily identified phenotypically (Quinn et al. 2011) and kept under refrigeration (4–8 °C) until further mass spectrometry analysis.

Filamentous fungi were identified based on the macromorphological structures of the colonies isolated on Sabouraud-dextrose agar; micromorphological analysis via microscopy of microculture slides on potato-dextrose agar; and phenotypic tests, including fermentation and assimilation of substrates. Species identification was based on taxonomic keys (Liu 2011, Petrini & Petrini 2013).

Umbilical infections were defined as ≥ 5 colony-forming units (CFUs) (Martins et al. 2023).

Mass spectrometry-based identification. All bacterial and yeast isolates were subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis on a Bruker Autoflex (Bruker Daltonik™, Bremen, Germany) equipment under 337 nm nitrogen laser conditions using FlexControl 3.3 software (Bruker Daltonik™, Bremen, Germany). The spectra were analyzed between 2,000 and 20,000 m/z using MALDI Biotyper 2.0 software (Bruker Daltonik™, Bremen, Germany) with default settings, and identification at the genus and species levels was considered ≥ 1.7 and ≥ 2.0, respectively (Gonçalves et al. 2014). All results that returned as "not identified" were subjected to one retest.

***In vitro* antimicrobial susceptibility testing.** The bacterial isolates were subjected to *in vitro* antimicrobial susceptibility testing using the disk diffusion method. Readings were carried out in accordance with the recommendations of the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI 2024).

Nine drugs belonging to six different antimicrobial groups, represented by drugs used in therapeutic approaches for lambs were tested as follows: (1) aminoglycosides (gentamicin 10 µg), (2) amphenicols (florfenicol 30 µg), (3) beta-lactams and derivatives (ampicillin 10 µg, amoxicillin with clavulanic acid 30 µg, ceftiofur 30 µg), (4) fluoroquinolones (enrofloxacin 5 µg, marbofloxacin 5 µg), (5) sulfonamides (trimethoprim/sulfamethoxazole 25 µg), and (6) tetracyclines (tetracycline 30 µg) (Constable et al. 2017, Altan et al. 2019, Lianou & Fthenakis 2022).

Bacteria were considered multidrug-resistant when isolates exhibited resistance to ≥ 3 antimicrobials from different classes (Magiorakos et al. 2012).

Statistical analysis. The associations between the occurrence of different agents and the clinical severity scores of umbilical infections were evaluated using the Kruskal-Wallis test, which compared the medians of clinical severity scores within each group or pathogen, with particular attention to case severity. The groups or microorganisms of special interest were subdivided as follows: non-*Escherichia coli* enteric bacteria, *E. coli*, *Pseudomonas* spp., enterococci, staphylococci, streptococci, actinomycetes, and miscellaneous. Dunn's post hoc test was performed for statistically significant combinations. The statistical significance of the percentage among severity scores was evaluated with a one-way chi-square test. Fisher's exact test with the Bonferroni correction was used to evaluate the death rate between the scores. Statistical differences were considered when p-values < 0.05.

RESULTS

Microorganisms. Among the 128 animals sampled, 44.5% (57/128) had mild umbilical infection (score 1), 35% (45/128) had moderate umbilical infection (score 2), and 20.5% (26/128) had severe umbilical infection (score 3). In addition to umbilical infection signs, 16.5% (21/128) of the lambs had diarrhea, 15% (19/128) had pneumonia, 8.5% (11/128) had septicemic signs (fever, tachycardia, respiratory distress, dyspnea), and 7% (9/128) had polyarthritis. Furthermore, 60 animals (60/128, 47%) had clinical signs compatible with complications of umbilical infections (i.e., pneumonia, arthritis, sepsis), and 58% (35/60) of them progressed to death. The p-value for the score distribution was 0.0033. The death rates among different scores were statistically different: 5.26% (3/57) for score 1, 26.66% (12/45) for score 2, and 76.92% (20/26) for score 3 ($p < 0.001$).

Isolation of bacteria and fungi (yeasts). A total of 214 microorganisms (among bacteria and yeasts) were isolated from 128 umbilical infection samples (Table 1).

The main microorganisms identified by mass spectrometry in diseased umbilical samples were enterobacteria and other Gram-negative bacteria: *Escherichia coli* (24/214, 11.21%), *Serratia marcescens* (8/214, 3.74%), *Raoultella terrigena* (7/214, 3.27%), *Aeromonas hydrophila* (6/214, 2.80%), *Pseudomonas koreensis* (6/214, 2.80%), *Enterobacter kobei* (6/214, 2.80%), and *Hafnia alvei* (5/214, 2.34%). Among all the isolates, 12.62% (27/214) were not identified by MALDI-TOF MS (Table 1).

Among the 128 diseased lambs, 59 umbilical samples had isolation of microorganisms in pure cultures, and the most common bacteria were *E. coli* (6/59, 10.17%), *S. marcescens* (4/59, 6.78%), *P. koreensis* (3/59, 5.08%), and *Providencia rettgeri* (3/59, 5.08%) (Table 2).

In 69 diseased umbilical samples (69/128, 53.9%), combined infections were observed, with the isolation of 57 different microorganism associations. The most common

Table 1. Prevalence of microorganisms isolated from umbilical infections of 128 lambs identified by mass spectrometry* (2022–2023)

Microorganism	N	%
Escherichia coli	24	11.21
Serratia marcescens	8	3.74
Enterococcus faecium	7	3.27
Raoultella terrigena	7	3.27
Aeromonas hydrophila	6	2.80
Pseudomonas koreensis	6	2.80
Enterobacter kobei	6	2.80
Hafnia alvei	5	2.34
Klebsiella oxytoca	5	2.34
Pseudomonas sp.	5	2.34
Enterococcus faecalis	4	1.87
Providencia rettgeri	4	1.87
Pseudomonas rhodesiae	4	1.87
Staphylococcus sciuri	4	1.87
Candida krusei	3	1.40
Enterobacter xiangfangensis	3	1.40
Enterococcus casseliflavus	3	1.40
Pseudomonas corrugata	3	1.40
Pseudomonas extremorientalis	3	1.40
Pseudomonas mendocina	3	1.40
Pseudomonas stutzeri	3	1.40
Rahnella aquatilis	3	1.40
Acinetobacter lwoffii	2	0.93
Enterobacter asburiae	2	0.93
Massilia oculi	2	0.93
Pantoea sp.	2	0.93
Proteus mirabilis	2	0.93
Pseudomonas oleovorans	2	0.93
Pseudomonas chlororaphis	2	0.93
Pseudomonas jessenii	2	0.93
Cellulosimicrobium cellulans	2	0.93
Enterococcus hirae	2	0.93
Pseudomonas putida	2	0.93
Streptococcus dysgalactiae	2	0.93
Others**	44	24.30
Not identified	27	12.62
TOTAL	214	100

* MALDI-TOF (MS) = Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; N = number of isolates; % = frequency of isolates; ** *Achromobacter mucicolens* (n = 1), *Acinetobacter radioresistens* (n = 1), *Aerococcus viridans* (n = 1), *Aeromonas jandaei* (n = 1), *Aeromonas veronii* (n = 1), *Arthrobacter arilaitensis* (n = 1), *Arthrobacter gandavensis* (n = 1), *Arthrobacter globiformis* (n = 1), *Arthrobacter koreensis* (n = 1), *Bacillus cereus* (n = 1), *Bacillus* sp. (n = 1), *Bordetella petrii* (n = 1), *Citrobacter koseri* (n = 1), *Diutina catenulata* (n = 1), *Enterobacter cancerogenus* (n = 1), *Enterobacter cloacae* (n = 1), *Enterococcus gallinarum* (n = 1), *Enterococcus gilvus* (n = 1), *Escherichia hermannii* (n = 1), *Escherichia vulneris* (n = 1), *Exiguobacterium mexicanum* (n = 1), *Klebsiella aerogenes* (n = 1), *Kluyvera ascorbata* (n = 1), *Kluyvera cryocrescens* (n = 1), *Leclercia adecarboxylata* (n = 1), *Lelliottia amnigena* (n = 1), *Oceanobacillus profundus* (n = 1), *Proteus hauseri* (n=1), *Proteus penneri* (n = 1), *Proteus vulgaris* (n = 1), *Pseudomonas azotoformans* (n = 1), *Pseudomonas brassicacearum* (n = 1), *Pseudomonas flavescens* (n = 1), *Pseudomonas gessardii* (n = 1), *Pseudomonas graminis* (n = 1), *Pseudomonas synxantha* (n = 1), *Pseudomonas trivialis* (n = 1), *Pseudomonas umsongensis* (n = 1), *Salmonella* sp. (n = 1), *Siccibacter turicensis* (n = 1), *Staphylococcus equorum* (n = 1), *Staphylococcus xylosus* (n = 1), *Streptococcus salivarius* (n = 1), *Wickerhamomyces anomalus* (n = 1).

associations were represented by *Enterococcus faecium* + *E. coli* (4/69, 5.8%), *H. alvei* + *Klebsiella oxytoca* (2/69, 2.9%), and *Pseudomonas* sp. + *Candida krusei* (2/69, 2.9%) (Table 3).

Among all 128 diseased umbilical samples, 3.9% (5/128) were negative for microbial isolation.

Scores of clinical severity. Among all lambs with umbilical infections, 44% (56/128), 35% (45/128), and 21% (27/128) presented mild (score 1), moderate (score 2), and severe (score 3) scores of clinical severity, respectively.

Among Gram-negative bacteria, a predominance of *E. coli* and *Pseudomonas* spp. was observed. Among infections caused by *E. coli*, 17% (4/24) presented mild clinical score severity, 50% (12/24) presented moderate scores, and 33% (8/24) presented severe scores. In turn, among infections caused by *Pseudomonas* spp., 43.2% (19/44) revealed mild clinical score severity, 34.1% (15/44) had moderate scores, and 22.7% (10/44) had severe scores.

Table 2. Isolation of microorganisms in pure culture in 128 neonatal lambs with omphalopathies, identified by mass spectrometry* (2022–2023)

Microorganism	N	%
Escherichia coli	6	10.17
Serratia marcescens	4	6.78
Pseudomonas koreensis	3	5.08
Providencia rettgeri	3	5.08
Enterobacter asburiae	2	3.39
Pseudomonas mendocina	2	3.39
Pseudomonas oleovorans	2	3.39
Pseudomonas rhodesiae	2	3.39
Pseudomonas sp.	2	3.39
Pseudomonas stutzeri	2	3.39
Aeromonas hydrophila	1	1.69
Aeromonas veronii	1	1.69
Arthrobacter globiformis	1	1.69
Bacillus sp.	1	1.69
Enterobacter kobei	1	1.69
Enterobacter xiangfangensis	1	1.69
Enterococcus casseliflavus	1	1.69
Enterococcus faecalis	1	1.69
Enterococcus gallinarum	1	1.69
Enterococcus hirae	1	1.69
Escherichia vulneralis	1	1.69
Pantoea sp.	1	1.69
Proteus mirabilis	1	1.69
Pseudomonas chlororaphis	1	1.69
Pseudomonas corrugata	1	1.69
Pseudomonas graminis	1	1.69
Pseudomonas jessenii	1	1.69
Pseudomonas umsongensis	1	1.69
Raoultella terrigena	1	1.69
Salmonella sp.	1	1.69
Not identified	6	10.17
Negative sample	5	8.47
TOTAL	59	100

* MALDI-TOF MS = Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; N = number of isolates.

Table 3. Prevalence of microorganisms isolated in combined infections in 128 lambs with omphalopathies identified by mass spectrometry* (2022–2023)

Microorganisms	N	%
Enterococcus faecium + *Escherichia coli*	4	5.8
Hafnia alvei + *Klebsiella oxytoca*	2	2.9
Pseudomonas sp. + *Candida krusei*	2	2.9
E. coli + Not identified	2	2.9
Aeromonas hydrophila + Not identified	2	2.9
Achromobacter mucicolens + *E. faecium* + *Staphylococcus sciuri* + *Wickerhamomyces anomalus*	1	1.45
Acinetobacter lwoffii + Not identified	1	1.45
A. lwoffii + *Acinetobacter radioresistens* + Not identified	1	1.45
Aerococcus viridans + *E. coli* + *Diutina catenulata*	1	1.45
A. hydrophila + *Kluyvera ascorbata*	1	1.45
A. hydrophila + *Enterococcus faecalis* + *E. faecium*	1	1.45
A. hydrophila + *E. coli*	1	1.45
Aeromonas jandaei + Not identified	1	1.45
Arthrobacter arilaitensis + Not identified	1	1.45
Arthrobacter gandavensis + *Pseudomonas stutzeri*	1	1.45
Arthrobacter koreensis + *Bacillus cereus* + *Enterococcus casseliflavus* + Not identified	1	1.45
Bordetella petrii + *Cellulosimicrobium cellulans* + *S. sciuri*	1	1.45
Cellulosimicrobium cellulans + Not identified	1	1.45
Citrobacter koseri + *E. coli*	1	1.45
Enterobacter cancerogenus + *Klebsiella aerogenes*	1	1.45
Enterobacter cloacae + *S. sciuri*	1	1.45
Enterobacter kobei + *E. casseliflavus* + *Pseudomonas chlororaphis*	1	1.45
E. kobei + *E. faecalis* + *Staphylococcus equorum*	1	1.45
E. kobei + *Leclercia adecarboxylata* + *Pseudomonas corrugata*	1	1.45
Other associations**	38	55.05
TOTAL	69	100

* MALDI-TOF MS = Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; N = number of associations, % = frequency of associations; ** *Enterobacter kobei* + not identified (n = 1), *E. kobei* + *P. corrugata* (n = 1), *Enterobacter xiangfangensis* + *H. alvei* + *K. oxytoca* (n = 1), *E. xiangfangensis* + *Pseudomonas rhodesiae* + *Raoultella terrigena* (n = 1), *E. faecalis* + *Serratia marcescens* + not identified (n = 1), *E. faecium* + *Oceanobacillus profundus* + not identified (n = 1), *Enterococcus gilvus* + *Exiguobacterium mexicanum* + *Kluyvera cryocrescens* (n = 1), *Enterococcus hirae* + *Pseudomonas putida* + not identified (n = 1), *Escherichia hermannii* + *E. coli* (n = 1), *E. coli* + *Proteus penneri* (n = 1), *E. coli* + *Pseudomonas aeruginosa* (n = 1), *E. coli* + *Pseudomonas brassicacearum* (n = 1), *E. coli* + *Pseudomonas flavescens* (n = 1), *E. coli* + *P. koreensis* (n = 1), *E. coli* + *Pseudomonas koreensis* + *R. terrigena (n = 1), E. coli* + *Siccibacter turicensis* (n = 1), *E. coli* + *Streptococcus dysgalactiae* (n = 1), *H. alvei* + not identified (n = 1), *H. alvei* + *R. terrigena* (n = 1), *K. oxytoca* + *Pantoea* sp. + *S. marcescens* (n = 1), *K. oxytoca* + *P. koreensis* (n = 1), *Lelliottia amnigena* + *Pseudomonas* sp. (n = 1), *Massilia oculi* + not identified (n = 1), *M. oculi* + *S. sciuri* + *C. krusei* + not identified (n = 1), *Proteus hauseri* + *S. marcescens* (n = 1), *Proteus mirabilis* + *S. marcescens* (n = 1), *Proteus vulgaris* + *R. terrigena* (n = 1), *Providencia rettgeri* + *R. terrigena* (n = 1), *Pseudomonas azotoformans* + *Rahnella aquatilis* (n = 1), *Pseudomonas extremorientalis* + *R. aquatilis* (n = 1), *P. extremorientalis* + *R. terrigena* + not identified (n = 1), *P. extremorientalis* + *P. rhodesiae* + *R. aqualitis* (n = 1), *Pseudomonas gessardii* + not identified (n = 1), *Pseudomonas jessenii* + *Pseudomonas mendocina* (n = 1), *Pseudomonas putida* + *Streptococcus salivarius* (n = 1), *Pseudomonas synxantha* + *Pseudomonas trivialis* (n = 1), *Staphylococcus xylosus* + not identified (n = 1), *Streptococcus dysgalactiae* + not identified (n = 1).

In 41 lambs without apparent signs of umbilical infections, 116 microorganisms were identified using mass spectrometry (Table 4). Among these animals, the main microorganisms isolated were *Desemzia incerta* (14/116, 12.1%), *Pantoea* sp. (8/116, 6.9%), *Pantoea agglomerans* (5/116, 4.3%), *P. koreensis* (4/116, 3.4%), *Pseudomonas stutzeri* (4/116, 3.4%), *Serratia fonticola* (4/116, 3.4%) and *Staphylococcus succinus* (4/116, 3.4%).

A wide range of microorganisms was identified in association with 41 lambs without apparent signs of umbilical infection (Table S1), where 36 different microorganism associations were found. Among these animals, only one (1/41, 2.4%) was negative for microbial isolation.

Strict anaerobic bacteria (e.g., *Clostridium* sp.) were not isolated from umbilical infections or from apparently

Table 4. Prevalence of microorganisms isolated from 41 lambs without apparent signs of umbilical infections, identified by mass spectrometry* (2022–2023)

Microorganism	N	%
Desemzia incerta	14	12.1
Pantoea sp.	8	6.9
Pantoea agglomerans	5	4.3
Pseudomonas koreensis	4	3.4
Pseudomonas stutzeri	4	3.4
Serratia fonticola	4	3.4
Staphylococcus succinus	4	3.4
Escherichia coli	3	2.6
Nocardiopsis dassonvillei	3	2.6
Pseudomonas chlororaphis	3	2.6
Pseudomonas flavescens	3	2.6
Acinetobacter lwoffii	2	1.7
Aeromonas hydrophila	2	1.7
Enterobacter kobei	2	1.7
Exiguobacterium mexicanum	2	1.7
Proteus penneri	2	1.7
Providencia rettgeri	2	1.7
Pseudomonas azotoformans	2	1.7
Pseudomonas fulva	2	1.7
Pseudomonas sp.	2	1.7
Serratia marcescens	2	1.7
Staphylococcus aureus	2	1.7
Staphylococcus xylosus	2	1.7
Others**	25	21.6
Not identified	12	10.3
TOTAL	116	100

* MALDI-TOF MS = Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; N = number of isolates, % = frequency of isolates; ** *Aerococcus viridans* (n = 1), *Arthrobacter gandavensis* (n = 1), *Arthrobacter koreensis* (n = 1), *Bacillus megaterium* (n = 1), *Citrobacter braakii* (n = 1), *Curtobacterium* sp. (n = 1), *Enterococcus faecium* (n = 1), *Exiguobacterium* sp. (n = 1), *Kerstersia gyiorum* (n = 1), *Klebsiella pneumoniae* (n = 1), *Klebsiella variicola* (n = 1), *Lactococcus garvieae* (n = 1), *Paenibacillus provencensis* (n = 1), *Pantoea ananatis* (n = 1), *Planococcus glaciei* (n = 1), *Proteus vulgaris* (n = 1), *Pseudomonas corrugata* (n = 1), *Pseudomonas fluorescens* (n = 1), *Pseudomonas jessenii* (n = 1), *Pseudomonas protegens* (n = 1), *Pseudomonas straminea* (n = 1), *Pseudomonas vancouverensis* (n = 1), *Serratia liquefaciens* (n = 1), *Serratia nematodiphila* (n = 1), *Solibacillus silvestris* (n = 1).

healthy animal samples. Filamentous fungi were isolated from 13 umbilical infection samples as follows: *Aspergillus* sp. (5/13, 38.5%), *Aspergillus niger* (1/13, 7.7%), *Aspergillus clavatus* (1/13, 7.7%), *Mucor* sp. (1/13, 7.7%), and *Geotrichum* sp. (1/13, 7.7%). Four filamentous fungi were not identified (4/13, 30.7%).

***In vitro* antimicrobial susceptibility testing.** Table 5 summarizes the *in vitro* antimicrobial susceptibility profiles of the main families of bacteria identified in lambs with umbilical infections. For the enterobacterial group, marbofloxacin (77/87, 88%), gentamicin (67/87, 77%), and sulfamethoxazole/trimethoprim (66/87, 76%) were the most effective drugs. In contrast, the isolates presented high resistance rates to ampicillin (66/87, 75%), amoxicillin/clavulanic acid (43/87, 49.5%), and florfenicol (41/87, 47%).

Marbofloxacin was the most effective drug against *Pseudomonas* spp. (42/44, 95.45%), whereas these isolates were resistant mainly to sulfamethoxazole/trimethoprim (20/44, 45%).

Among the enterococcus strains, amoxicillin/clavulanic acid was the most effective drug (13/17, 76%), whereas the isolates presented a high resistance rate to enrofloxacin (13/17, 76%).

Marbofloxacin (6/6, 100%), ceftiofur (5/6, 83%), and gentamicin (5/6, 83%) were the most effective drugs for the staphylococcal group. Conversely, despite the small number of staphylococci tested, the isolates were resistant mainly to ampicillin (3/6, 50%).

Streptococci showed 100% (3/3) susceptibility to amoxicillin/clavulanic acid and ceftiofur, whereas 67% (2/3) of this bacterial group were resistant to enrofloxacin, despite the small number of tested streptococci. Bacterial multidrug resistance was observed in 20% (36/182) of the isolates from umbilical infection samples. The most common multidrug-resistant species were *E. coli* (10/24, 41%), *E. faecium* (5/7, 71%), and *S. marcescens* (2/8, 25%). Among the 36 multidrug-resistant isolates, 27 (27/36, 75%) were Gram-negative bacteria.

Table 5. *In vitro* antimicrobial susceptibility profile (disc diffusion method) in 182 bacteria isolated from umbilical infections of 128 lambs

Class	Antimicrobial agent	Families (NCBI 2024)														
		Enterobacteriaceae[A] Erwiniaceae[B] Hafniaceae[C] Morganellaceae[D] Yersiniaceae[E] (n = 87)			Aeromonadaceae[F] Alcaligenaceae[G] Oxalobacteraceae[H] Pseudomonadaceae[I] (n =56)			Moraxellaceae[J] (n =3)			Bacillaceae[K] Staphylococcaceae[L] (n = 9)			Aerococcaceae[M] Enterococcaceae[N] Micrococcaceae[O] Promicromonosporaceae[P] Streptococcaceae[Q] (n = 27)		
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)
Aminoglycosides	Gentamicin[a]	77	14	9	87.5	3.5	9	66.67	0	33.33	77.8	11.1	11.1	-	-	-
Amphenicols	Florfenicol[b]	31	22	47	-	-	-	100	0	0	-	-	-	22	15	63
Cephalosporins	Ceftiofur[b]	33	48	19	-	-	-	0	67	33	66.7	0	33.3	22.2	7.4	70.4
Fluorquinolones	Enrofloxacin[a]	39	50	11	55	36	9	33.4	33.3	33.3	11.1	77.8	11.1	18.5	18.5	63
	Marbofloxacin[c]	88	1	11	92.86	3.57	3.57	-	-	-	77.8	11.1	11.1	37	22.2	51.8
Beta-lactams	Amoxicillin/clavulanic acid[c]	49.5	1	9	-	-	-	67	0	33	77.8	0	22.2	85	0	15
	Ampicilin[d]	23	2	75	-	-	-	67	0	33	44.5	22.2	33.3	59.2	7.5	33.3
Sulfonamides	Sulfamethoxazole/ trimethoprim	76	5	19	55	20	25	-	-	-	55.6	22.2	22.2	22.2	0	77.8
Tetracyclines	Tetracycline[e]	64	3	33	-	-	-	33	0	67	66.7	0	33.3	55.5	14.8	29.7

S = Susceptible, I = intermediate, R = resistant; [A] *Citrobacter koseri* (n = 1), *Enterobacter asburiae* (n = 2), *Enterobacter cancerogenus* (n = 1), *Enterobacter cloacae* (n = 1), *Enterobacter kobei* (n = 6), *Enterobacter xiangfangensis* (n = 3), *Escherichia coli* (n = 24), *Escherichia hermannii* (n = 1), *Escherichia vulneris* (n = 1), *Klebsiella aerogenes* (n = 1), *Klebsiella oxytoca* (n = 5), *Kluyvera ascorbata* (n = 1), *Kluyvera cryocrescens* (n = 1), *Leclercia adecarboxylata* (n = 1), *Lelliottia amnigena* (n = 1), *Raoultella terrigena* (n = 7), *Salmonella* sp. (n = 1), *Siccibacter turicensis* (n = 1); [B] *Pantoea* sp. (n = 2); [C] *Hafnia alvei* (n = 5); [D] *Providencia rettgeri* (n=4), *Proteus hauseri* (n = 1), *Proteus mirabilis* (n = 2), *Proteus penneri* (n = 1), *Proteus vulgaris* (n = 2); [E] *Rahnella aquatilis* (n = 3), *Serratia marcescens* (n = 8); [F] *Achromobacter mucicolens* (n = 1), *Bordetella petrii* (n = 1); [G] *Aeromonas hydrophila* (n = 6), *Aeromonas jandaei* (n = 1), *Aeromonas veronii* (n = 1); [H] *Massilia oculi* (n = 2); [I] *Pseudomonas aeruginosa* (n = 1), *Pseudomonas azotoformans* (n = 1), *Pseudomonas brassicacearum* (n = 1), *Pseudomonas chlororaphis* (n = 2), *Pseudomonas corrugata* (n = 3), *Pseudomonas extremorientalis* (n = 3), *Pseudomonas flavescens* (n = 1), *Pseudomonas gessardii* (n = 1), *Pseudomonas graminis* (n = 1), *Pseudomonas jessenii* (n = 2), *Pseudomonas koreensis* (n = 6), *Pseudomonas mendocina* (n = 3), *Pseudomonas oleovorans* (n = 2), *Pseudomonas putida* (n = 2), *Pseudomonas rhodesiae* (n = 4), *Pseudomonas* sp. (n = 5), *Pseudomonas stutzeri* (n = 3), *Pseudomonas synxantha* (n = 1), *Pseudomonas trivialis* (n = 1), *Pseudomonas umsongensis* (n = 1); [J] *Acinetobacter lwoffii* (n = 2), *Acinetobacter radioresistens* (n = 1); [K] *Bacillus* sp. (n = 1), *Exiguobacterium mexicanum* (n = 1), *Oceanobacillus profundus* (n = 1); [L] *Staphylococcus equorum* (n = 1), *Staphylococcus sciuri* (n = 4), *Staphylococcus xylosus* (n = 1); [M] *Aerococcus viridans* (n = 1); [N] *Enterococcus casseliflavus* (n = 2), *Enterococcus faecalis* (n = 4), *Enterococcus faecium* (n = 7), *Enterococcus gallinarum* (n = 1), *Enterococcus gilvus* (n = 1), *Enterococcus hirae* (n = 2); [O] *Arthrobacter arilaitensis* (n = 1), *Arthrobacter gandavensis* (n = 1), *Arthrobacter globiformis* (n = 1), *Arthrobacter koreensis* (n = 1); [P] *Cellulosimicrobium cellulans* (n = 2); [Q] *Streptococcus dysgalactiae* (n = 2), *Streptococcus salivarius* (n = 1); [a] Breakpoints for livestock (horses) (CLSI 2024), [b] breakpoints for livestock (swine) (CLSI 2024), [c] breakpoints for companion animals (dogs) (CLSI 2024), [d] breakpoints for livestock (cattle) (CLSI 2024), [e] breakpoints for humans (CLSI 2024).

Among the microorganisms isolated from lambs without apparent signs of umbilical infections, gentamicin (26/26, 100%) and marbofloxacin (26/26, 100%) were the most effective drugs for *Pseudomonas* spp. Gentamicin (8/8, 100%) and marbofloxacin (8/8, 100%) were the most effective drugs in the staphylococcal group, whereas the isolates showed higher resistance to sulfamethoxazole/trimethoprim (3/8, 37.5%). For the enterobacterial group, gentamicin (31/32, 97%), florfenicol (30/32, 94%), and marbofloxacin (30/32, 94%) were highly effective. In the miscellaneous group, tetracycline (33/35, 94%), amoxicillin/clavulanic acid (32/35, 91%), ampicillin (32/35, 91%), and florfenicol (32/35, 91%) were the most effective drugs, while the isolates showed high resistance to enrofloxacin (18/35, 51%). The prevalence of multidrug resistance among bacteria isolated from lambs without apparent umbilical signs was 5.9% (6/101).

Statistical analysis. A significant difference ($p = 0.0039$) was observed between the isolation of *E. coli* and moderate and severe clinical scores. In pairwise comparisons, the median CSS scores were higher for the *E. coli* group than for other specific groups of microorganisms studied.

DISCUSSION

Umbilical infections in domestic ruminants possess a multifactorial etiology, although they predominantly have an infectious origin (Fordyce et al. 2018, Palm et al. 2022, Martins et al. 2023). The disease has been related to significant economic impacts, particularly due to systemic complications (Windeyer et al. 2014, Fordyce et al. 2018, Tora et al. 2021). These infections occur via an ascending route, secondary to contact of the navel with the environment, or from the own navel microbiota (Windeyer et al. 2014, Constable et al. 2017, Fordyce et al. 2018). The high prevalence of umbilical infections in newborn lambs is likely related to the impaired immunity of the animals due to the low ingestion or poor quality of colostrum and inadequate antisepsis of the umbilical cord in the first days after birth (Giadinis et al. 2009, Gunn et al. 2015, Ratanapob et al. 2020).

In the current study, the high bacterial complexity was identified in diseased lambs, with a predominance of the enterobacteria group, particularly *Escherichia coli*. Enterobacteria constitute the main isolated group of microorganisms in animals with umbilical infection, in pure culture or association. The predominance of enterobacteria and other Gram-negative pathogens infecting the umbilical cord of lambs in the present study is consistent with similar studies conducted on calves in Colombia (Cardona et al. 2011), Iran (Faradonbeh & Faradonbeh 2016), and Brazil (Martins et al. 2023). This finding can be attributed to the high exposure of the navel in the first days postpartum to environmental pathogens (i.e., soil, feces, organic matter, and water), especially *E. coli* (Quinn et al. 2011, Steerforth & Van Windem 2018).

E. coli is characterized by high versatility and adaptability to different biomes, possesses high genetic diversity, clonal variety, complexity of virulence factors, and resistance to conventional antimicrobials (Jang et al. 2017, Denamur et al. 2021). Pathogenic *E. coli* is classically subdivided into enteric and extraenteric (ExPEC), and presents a wide variety of virulence factors, including toxins, fimbriae/adhesins, invasines, iron-uptake systems (siderophores), and mechanisms of serum resistance (Jang et al. 2017, Sarowska et al. 2019, Denamur et al. 2021). In this context, a recent study in Brazil investigated virulence genes related to ExPEC from *E. coli* isolated from calves with umbilical infections and their association with the clinical severity scores of the cases, and revealed a predominance of serum resistance genes (*traT* and *ompT*) and a high frequency of severe cases (score 3). However, the specific gene profile assigned to the disease was not identified (Martins et al. 2023).

The purpose of standardizing clinical severity scores for cases of umbilical infections in calves has been proposed over the last two decades (Steerforth & Van Winden 2018, Martins et al. 2023). In turn, no studies have investigated the severity scores of umbilical infections in lambs, a fact that may be considered a main motivation of the present study. In this regard, we adapted, for the first time, the clinical severity scoring of umbilical infections in bovine calves for use in lambs, and a significant difference ($p = 0.0039$) was found between the isolation of *E. coli* and moderate and severe clinical scores.

Lipopolysaccharides (LPS) are major components of the external membrane of Gram-negative bacteria, including *E. coli*. The lipid A fraction of LPS is liberated with the death of *E. coli* by the immune response of hosts. The liberation of lipid A increases vascular permeability and activates strong inflammatory precursors, such as tumoral necrosis factor (TNF) and interleukins (which increase leucocyte chemotaxis), which induce fever, respiratory distress, high cardiac rate, decubitus, and death, caused by sepsis and endotoxic shock (Schukken et al. 2012). In fact, the complexity of the inflammatory effects caused by *E. coli*'s LPS is probably related to the higher scores of clinical severities (2 and 3) observed in the present study in diseased lambs.

Enterobacter, Hafnia, Klebsiella, Serratia, Providencia, Proteus, and *Pantoea* species represented other enteric Gram-negative bacteria found in the diseased lambs studied. This finding reinforces the etiological complexity and impact of enteric bacteria, which have an environmental origin, as primary agents of umbilical infections in lambs (Faradonbeh & Faradonbeh 2016). Similarly to calves, enterobacterial species are likely acquired through high exposure of the umbilical area of lambs to organic matter, feces, soil, dirt, water, and from contaminated utensils in the immediate environment of the animals (Martins et al. 2023).

Pseudomonas species are well-known opportunistic bacteria associated with a wide range of clinical infections in domestic animals, e.g., genitourinary, pulmonary, umbilical, bone, ear canal, and enteric disorders (Quinn et al. 2011). This microorganism is widely distributed in animal farm environments (Quinn et al. 2011), favoring opportunistic infections, which are mainly associated with watery biomes (Schauer et al. 2021), including umbilical infections in ruminants (Cardona et al. 2011, Faradonbeh & Faradonbeh 2016, Schauer et al. 2021). In this study, over 20 *Pseudomonas* species were identified in diseased lambs, some of which were probably described for the first time as primary agents related to umbilical infections in lambs (e.g., *Pseudomonas azotoformans, Pseudomonas brassicacearum, Pseudomonas corrugata, Pseudomonas extremorientalis,* and *Pseudomonas stutzeri*). This finding could be attributed to the use of the mass

spectrometry technique for diagnosis, which presents a high ability to distinguish bacterial species (Gonçalves et al. 2014).

Staphylococci and streptococci are classified as a complex group of Gram-positive cocci that inhabit the skin, conjunctiva, and mucous membranes of animals and humans and are related to a variety of purulent clinical manifestations (Quinn et al. 2011, McCulloch & Mamizuca 2015). Previous studies with neonatal lambs (Rutherford et al. 2014, Smistad et al. 2020) and calves (Cardona et al. 2011, Martins et al. 2023) described staphylococci and streptococci species as frequent pathogens in umbilical infections. Conversely, in the present study, *Staphylococcus* and *Streptococcus* species were isolated at low frequencies. These discrepancies in the frequency of umbilical infections could be attributed to the differences in the management practices adopted, especially regarding antisepsis procedures for the umbilical cord in the first days of life (Faradonbeh & Faradonbeh 2016, Salci et al. 2017, Steerforth & Van Winden 2018). Despite the low frequency, the isolation of staphylococci and streptococci species as primary agents of umbilical infections in lambs emphasizes the need for preventive/control measures against infectious omphalopathies on farms studied, particularly antisepsis approaches involving the umbilical cord (Martins et al. 2023), owing to the presence of these microorganisms in the skin and mucous membranes (Quinn et al. 2011), including the umbilical region of domestic ruminants (Martins et al. 2023).

Enterococcus species were other Gram-positive bacteria found in the sampled lambs. This bacterial group inhabits the digestive tract of animals and humans and is also present in the environment of farms (Quinn et al. 2011), causing opportunistic infections, including omphalopathies in calves (Martins et al. 2023). The identification of different *Enterococcus* species in diseased lambs reinforces the etiological complexity of umbilical disorders in domestic ruminants.

Rutherford et al. (2014), Ribeiro et al. (2015), and Constable et al. (2017) referred to *Trueperella pyogenes* as a frequent pathogen in umbilical infections of neonatal ruminants in the United Kingdom, Brazil, and the USA, respectively. However, in the present study, *T. pyogenes* was not isolated, which could be credited to the variations in husbandry practices, housing conditions, control of flies, and antisepsis approaches of the navel on the farms studied.

Even though fungal/yeast isolation was presented at low frequency and in coinfections with bacteria, it should also be considered in diagnostic approaches as a primary cause of umbilical infections in lambs, owing to the presence of these microorganisms in the farm environment (Quinn et al. 2011).

Most studies on the etiology of umbilical infections in lambs (Holmøy et al. 2012, Faradonbeh & Faradonbeh 2016) have been based on conventional methods involving phenotypic identification of the microorganisms (Giadinis et al. 2009, Ribeiro et al. 2015). Nonetheless, in the present study, the pathogens were identified at the species level using a molecular method based on mass spectrometry. The MALDI-TOF MS technique enables reliable identification of microorganisms in the current study, due to the high discrimination power to distinguish bacteria and yeasts at the species level (Tsuchida et al. 2020). In this regard, to our knowledge, many species of bacteria were identified for the first time in the current study as agents of umbilical infections of neonatal lambs, e.g., *Raoultella terrigena, Rahnella aquatilis, Massilia oculi, Achromobacter mucicolens, Acinetobacter radioresistens, Arthrobacter globiformis, Arthrobacter gandavensis, Kluyvera ascorbata, Kluyvera cryocrescens, Leclercia adecarboxylata, Siccibacter turicensis, Oceanobacillus profundus,* and *Wickerhamomyces anomalus*.

Among all animals studied, 3.9% of samples from clinical cases of umbilical infections were negative, which could be attributed to agents that need special conditions of isolation or aseptic inflammation, i.e., caused by trauma or parasites, which were not investigated in the present study (Constable et al. 2017).

In relation to lambs without apparent signs of umbilical infections, a predominance of Gram-positive bacteria was identified (Wei et al. 2023). *Desemzia incerta* was the most frequent microorganism isolated from animals without apparent signs of umbilical infection. It has been isolated from meat, fish, and dairy products (Batt & Tortorello 2014) and from the oral cavity of humans (Adhikari et al. 2017). Nonetheless, the lack of reports identifying *D. incerta* in sheep limits the evaluation of the impact of this bacterium on the microbiota of the umbilical cord of neonatal lambs.

For enterobacteria and *Pseudomonas* species isolated from diseased lambs, marbofloxacin exhibited high efficacy, whereas for *Streptococcus*, *Staphylococcus,* and *Enterococcus* species, gentamicin, amoxicillin/clavulanic acid, and ceftiofur showed moderate efficacy. These findings indicate that, in general, these antimicrobials may be considered options for the treatment of umbilical infections in lambs in the studied region.

In general, bacteria isolated from umbilical infections in lambs showed > 30% *in vitro* resistance to ampicillin, tetracycline, and sulfamethoxazole/trimethoprim. This result is expected because these drugs have been used in therapeutic approaches for domestic ruminants for more than 40 years, which may increase the selective pressure on multidrug-resistant isolates (Giguère et al. 2010). In addition, approximately 20% of the bacterial isolates were multidrug resistant, mainly the Gram-negative ones, which is considered a global emerging issue in animals and humans (WHO 2022, Donkor et al. 2023), reinforcing the need for proper antimicrobial use in therapeutic approaches in domestic animals, including the adoption of previous *in vitro* antimicrobial susceptibility tests prior to treatment procedures (Giguère et al. 2010).

Among all diseased lambs, data on outcomes were available for 47% of the animals, and many developed severe clinical signs (i.e., pneumonia, arthritis, sepsis). Subsequently, they died, probably due to complications secondary to umbilical infections (Dunne 2021). The high mortality related to umbilical infections in lambs is consistent with the findings of a similar study in Morocco (Chaarani et al. 1991). In this regard, a significant association ($p < 0.001$) was observed between moderate and severe scores of umbilical infections among lambs sampled, indicating that these high-severity scores are related to poor prognosis or fatal outcomes.

Considering the economic losses to farmers related to umbilical infections and the high mortality of neonatal lambs (Windeyer et al. 2014, Tora et al. 2021), it is necessary to adopt measures for control/prevention of the disease on the farms studied, especially adequate umbilical antisepsis of neonatal lambs and proper colostrum intake in the first hours of life (Windeyer et al. 2014, Faradonbeh & Faradonbeh 2016,

Rajamohan et al. 2025). In addition, preventive measures should be focused on the environment of lambs, e.g., daily feces and organic matter removal (avoiding excess humidity in pens), and providing a clean and dry place in husbandry, owing to the high occurrence of enterobacteria and other Gram-negative bacteria that inhabit the farm environment, which represent a high risk of diseases to the lambs, including umbilical infections.

The convenience sampling of apparently healthy lambs, the lack of outcome/complication data, the missing information on the age and gender of some animals, the use of phenotypic methods for fungal identification, and the non-investigation of the virulence factors in the *E. coli* isolates may be considered limitations of the current study.

CONCLUSIONS

A great complexity of bacterial species was identified by mass spectrometry in neonatal lambs with umbilical infections. *Escherichia coli* was the predominant agent in diseased neonatal lambs and was significantly associated with moderate and severe clinical severity scores. Likewise, a great complexity of bacteria was also found in the newborn lambs, without apparent umbilical signs. Additionally, 20% of bacteria isolated from umbilical infections were multidrug-resistant, indicating the need for rational use of antimicrobials in treating approaches to this disease. High mortality (58%) was observed in lambs with clinical complications of umbilical infections, highlighting the losses of the disease on farms.

This study contributes to molecular identification of the etiology, clinical severity scoring, and *in vitro* antimicrobial profile of umbilical infections in lambs, as well as microbiota identification of the umbilical cord of apparently healthy lambs.

Acknowledgments.- The authors thank the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq), Brazil, for research productivity fellowships (PQ) given to Márcio Garcia Ribeiro (#310345/2020-0).

Funding.- This research was financed, in part, by the "Fundação de Amparo à Pesquisa do Estado de São Paulo" (FAPESP), Brazil, grant 2021/15085-8.

Conflict of interest statement.- The authors declare that they have no conflicts of interest.

Credit author statement.- Conceptualization and writing – original draft: Thaís S. Bello, Márcio G. Ribeiro. Methodology and investigation: Thaís S. Bello; Formal analysis: Thaís S. Bello, Fábio R. Portilho. Sampling: Rodrigo G. Motta, Lorrayne M. Motta, Geraldo de Nardi, Rodolfo C. Souto, José A. Bastos. Statistical analysis: Fábio S. Possebon. Writing – review & editing: Thaís S. Bello, Rodrigo G. Motta, Lorrayne M. Motta, Geraldo de Nardi, Rodolfo C. Souto, José A. Bastos, Fábio R. Portilho, Fábio S. Possebon, Patrik L. Paz, Márcio G. Ribeiro. Validation, funding acquisition, project administration and supervision: Márcio G. Ribeiro.

Data availability statement.- All the authors confirm that the data supporting the findings of this study are available within the article and its supplementary material. In addition, raw data supporting this study's findings are available from the corresponding author upon reasonable request.

Editor-in-Chief.- Fabiano José Ferreira de Sant'Ana.

REFERENCES

Adhikari A, Kurella S, Banerjee P, Mitra A. Aerosolized bacteria and microbial activity in dental clinics during cleaning procedures. J Aerosol Sci 2017; https://doi.org/10.1016/j.jaerosci.2017.09.019

Altan F, Sayin Ipek DN, Corum O, Alp SY, Ipek P, Uney K. The effects of Mannheimia haemolytica and albendazole on marbofloxacin pharmacokinetics in lambs. Trop Anim Health Prod 2019; https://doi.org/10.1007/s11250-019-01980-5

Barry J, Bokkers EAM, Sayers R, Murphy JP, Boer IJM, Kennedy E. Effect of feeding single-dam or pooled colostrum on maternally derived immunity in dairy calves. J Dairy Sci 2022; https://doi.org/10.3168/jds.2021-20343

Batt CA, Tortorello M-L. Encyclopedia of Food Microbiology. 2nd ed. London: Academic Press; 2014.

Binns SH, Cox IJ, Rizvi S, Green LE. Risk factors for lamb mortality on UK sheep farms. Prev Vet Med 2002; https://doi.org/10.1016/S0167-5877(01)00255-0

Cardona ÁJ, Álvarez PJ, Arrieta BG. Aislamiento e identificación de agentes bacterianos productores de onfalitis en terneros del departamento de Córdoba. Rev U DCA Act Div Cient 2011; https://doi.org/10.31910/rudca.v14.n2.2011.779

Chaarani B, Robinson RA, Johnson DW. Lamb mortality in Meknes province (Morocco). Prev Vet Med 1991; https://doi.org/10.1016/0167-5877(91)90014-S

CLSI. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. 7th ed. CLSI VET01S, Clinical and Laboratory Standards Institute; 2024.

Constable PD, Hinchliff KW, Done SH, Grüenberg W. Veterinary medicine: a textbook of the diseases of cattle, horses, sheep, pigs, and goats. 11th ed. Vol. 2. Philadelphia: Saunders; 2017.

Denamur E, Clermont O, Bonacorsi S, Gordon D. The population genetics of pathogenic Escherichia coli. Nat Rev Microbiol 2021; https://doi.org/10.1038/s41579-020-0416-x

Donkor GY, Anderson GM, Stadler M, Tawiah PO, Orellano CD, Edwards KA, Dahl J-U. A novel ruthenium-silver based antimicrobial potentiates aminoglycoside activity against Pseudomonas aeruginosa. mSphere 2023; https://doi.org/10.1128/msphere.00190-23

Dunne K. Umbilical disinfection options in large animal neonates. Ir Vet J 2021; https://www.researchgate.net/publication/354022607_A_guide_to_umbilical_disinfection_options_in_large_animal_neonates

Faradonbeh YK, Faradonbeh MK. Evaluate the risk factors umbilical cord bacterial infection in calves in Shahrekord city. J Entomol Zool Stud 2016; https://www.entomoljournal.com/archives/2016/vol4issue2/PartC/3-6-78.pdf

Farooq U, Ahmed S, Liu G, Jiang X, Yang H, Ding J, Ali M. Biochemical properties of sheep colostrum and its potential benefits for lamb survival: a review. Anim Biotechnol 2024; https://doi.org/10.1080/10495398.2024.2320726

Fordyce AL, Timms LL, Stalder KJ, Tyler HD. Short communication: the effect of novel antiseptic compounds on umbilical cord healing and incidence of infection in dairy calves. J Dairy Sci 2018; https://doi.org/10.3168/jds.2017-13181

Giadinis ND, Loukopoulos P, Tsakos P, Kritsepi-Konstantinou M, Kaldrymidou E, Karatzias H. Illthrift in suckling lambs attributed to lung pyogranuloma formation. Vet Med Int 2009; https://doi.org/10.1136/vr.165.12.348

Giguère S, Prescott JF, Baggot JD, Walker RD, Dowling PM. Terapia Antimicrobiana em Medicina Veterinária. 4ª ed. São Paulo: Roca; 2010.

Gökçe E, Erdoğan HM. An epidemiological study on neonatal lamb health. Kafka Üniv Vet Fak Derg 2009; https://vetdergikafkas.org/uploads/pdf/pdf_KVFD_461.pdf

Gonçalves JL, Tomazi T, Barreiro JR, Braga PAC, Ferreira CR, Araújo Junior JP, Eberlin MN, Santos MV. Identification of *Corynebacterium* spp. isolated from bovine intramammary infections by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Vet Microbiol 2014; https://doi.org/10.1016/j.vetmic.2014.06.028

Gunn A, House JK, Chuck GM. Initial management and clinical investigation of neonatal ruminants, p. 286-301. In: Smith BP. Large Animal Internal Medicine. 5th ed. St. Louis: Elsevier Mosby; 2015.

Holmøy IH, Kielland C, Stubsjøen SM, Hektoen L, Waage S. Housing conditions and management practices associated with neonatal lamb mortality in sheep flocks in Norway. Prev Vet Med 2012; https://doi.org/10.1016/j.prevetmed.2012.06.007

Hordofa D, Abunna F, Megersa B, Abebe R. Incidence of morbidity and mortality in calves from birth to six months of age and associated risk factors on dairy farms in Hawassa city, southern Ethiopia. Heliyon 2021; https://doi.org/10.1016/j.heliyon.2021.e08546

Ismail ZB, Muhaffel MM. Dairy calf and replacement heifer mortality on a single intensively managed dairy farm in Jordan: a 3-year-long study (2016–2018). Open Vet J 2022; https://doi.org/10.5455/OVJ.2022.v12.i6.21

Jang J, Hur H-G, Sadowsky MJ, Byappanahalli MN, Yan T, Ishii S. Environmental *Escherichia coli*: ecology and public health implications—a review. J Appl Microbiol 2017; https://doi.org/10.1111/jam.13468

Lianou DT, Fthenakis GC. Use of antibiotics against bacterial infections on dairy sheep and goat farms: Patterns of usage and associations with health management and human resources. Antibiotics 2022; https://doi.org/10.3390/antibiotics11060753

Liu D. Molecular Detection of Human Fungal Pathogens. 2011; https://doi.org/10.1201/b11375

Magiorakos A-P, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect 2012; https://doi.org/10.1111/j.1469-0691.2011.03570.x

Martins LSA, Motta RG, Martinez AC, Orsi H, Hernandes RT, Rall VLM, Pantoja JCF, Nardi Júnior G, Ribeiro MG. Virulence-encoding genes related to extraintestinal pathogenic E. *coli* and multidrug resistant pattern of strains isolated from neonatal calves with different severity scores of umbilical infections. Microb Pathog 2023; https://doi.org/10.1016/j.micpath.2022.105861

McCulloch H, Mamizuca EM. Staplylococcus aureus. In: Trabulsi LR, Alterthum F. Microbiologia. 6ª ed. São Paulo: Atheneu; 2015.

Mee JF. Invited review: bovine neonatal morbidity and mortality—Causes, risk factors, incidences, sequelae and prevention. Reprod Domest Anim 2023; https://doi.org/10.1111/rda.14369

Palm HA, Thirumalapura NR, Boger LA, Ringler SR. An unusual outbreak of erysipelas on a goat farm in Pennsylvania. J Vet Diagn Invest 2022; https://doi.org/10.1177/10406387211072831

Petrini LE, Petrini O. Identifying moulds: a practical guide. Stuttgart: J Cramer; 2013.

Quinn PJ, Markey BK, Leonard FC, Fitzpatrick ES, Fanning S, Hartigan PJ. Veterinary Microbiology and Microbial Disease. 2nd ed. Oxford: Wiley-Blackwell; 2011. p.334-341.

Rajamohan S, Rajasekaran R, Krishnan P, Navlon RJP. Navel and joint ill, p. 495-500. In: Rana T. Elements of Reproduction and Reproductive Diseases of Goats. 2025; https://doi.org/10.1002/9781394190089.ch44

Ratanapob N, VanLeeuwen J, McKenna S, Wichtel M, Stryhn H, Rodriguez-Lecompte JC, Menzies P, Wichtel J. Management factors associated with perinatal lamb mortality in Prince Edward Island flocks. Prev Vet Med 2020; https://doi.org/10.1016/j.prevetmed.2020.105035

Ribeiro MG, Risseti RM, Bolaños CAD, Caffaro KA, Morais ACB, Lara GHB, Zamprogna TO, Paes AC, Listoni FJP, Franco MMJ. *Trueperella pyogenes* multispecies infections in domestic animals: a retrospective study of 144 cases (2002 to 2012). Vet Q 2015; https://doi.org/10.1080/01652176.2015.1022667

Rutherford SJ, Rycroft AN, Ridler AL. Sources of *Streptococcus dysgalactiae* in English and Welsh sheep flocks affected by infectious arthritis (joint ill). Vet Rec 2014; https://doi.org/10.1136/vr.101753

Salci H, Ozdemir Salci ES, Ozakin C, Seyrek Intas K. A brief study on hematological, sero-biochemical and microbiological results of umbilical lesions in calves. Int J Vet Sci 2017; https://www.ijvets.com/pdf-files/Volume-6-no-2-2017/86-89.pdf

Sarowska J, Futoma-Koloch B, Jama-Kmiecik A, Frej-Madrzak M, Ksiazczyk M, Bugla-Ploskonska G, Choroszy-Krol I. Virulence factors, prevalence and potential transmission of extraintestinal pathogenic *Escherichia coli* isolated from different sources: recent reports. Gut Pathog 2019; https://doi.org/10.1186/s13099-019-0290-0

Sato R, Yamada K, Yokoyama T, Tanimoto K, Takeuchi S, Tatsuzawa N, Nakui S, Satoh H, Fadul M, Steiner A. Partial hepatectomy for treatment of multiple liver abscess in a calf: a case report. BMC Vet Res 2021; https://doi.org/10.1186/s12917-021-02750-y

Schauer B, Wald R, Urbantke V, Loncaric I, Baumgartner M. Tracing mastitis pathogens—Epidemiological investigations of a *Pseudomonas aeruginosa* mastitis outbreak in an Austrian dairy herd. Animals 2021; https://doi.org/10.3390/ani11020279

Schukken Y, Chuff M, Moroni P, Gurjar A, Santisteban C, Welcome F, Zadoks R. The "other" gram-negative bacteria in mastitis: Klebsiella, Serratia, and more. Vet Clin North Am Food Anim Pract 2012; https://doi.org/10.1016/j.cvfa.2012.04.001

Scott LC, Menzies PI. Antimicrobial resistance and small ruminant veterinary practice. Vet Clin N Am Food Anim Pract 2011; https://doi.org/10.1016/j.cvfa.2010.10.015

Smistad M, Tollersrud TS, Austbø L, Porcellato D, Wolff C, Asal B, Phythian CJ, Oppegaard O, Jørgensen HJ. Molecular detection and genotype characterization of Streptococcus dysgalactiae from sheep flocks with outbreaks of infectious arthritis. Vet Microbiol 2021; https://doi.org/10.1016/j.vetmic.2021.109221

Smistad M, Wolff C, Tollersrud T, Tømmerberg V, Phythian C, Kampen AH, Jørgensen HJ. Flock-level risk factors for outbreaks of infectious arthritis in lambs, Norway 2018. Acta Vet Scand 2020; https://doi.org/10.1186/s13028-020-00561-z

Steerforth D-D, Van Winden S. Development of clinical sign-based scoring system for assessment of omphalitis in neonatal calves. Vet Rec 2018; https://doi.org/10.1136/vr.104213

Tora E, Abayneh E, Seyoum W, Shurbe M. Longitudinal study of calf morbidity and mortality on smallholder farms in southern Ethiopia. PLoS One 2021; https://doi.org/10.1371/journal.pone.0257139

Tsuchida S, Umemura H, Nakayama T. Current status of matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) in clinical diagnostic microbiology. Molecules 2020; https://doi.org/10.3390/molecules25204775

Tüfekci H, Sejian V. Stress factors and their effects on productivity in sheep. Animals 2023; https://doi.org/10.3390/ani13172769

Underwood WJ, Blauwiekel R, Delano ML, Gillesby R, Mischler SA, Schoell A. Biology and diseases of ruminants (sheep, goats, and cattle), p. 623-694. In: Fox JG, Anderson LC, Otto GM, Pritchett-Corning KR, Whary MT. Laboratory Animal Medicine. 2015; https://doi.org/10.1016/B978-0-12-409527-4.00015-8

Van Camp MB, Winder CB, Gomez DE, Duffield TF, Savor NK, Renaud DL. Evaluating the effectiveness of a single application of 7% iodine tincture umbilical dip as a prevention of infection of the external umbilical structures in dairy calves. J Dairy Sci 2022; https://doi.org/10.3168/jds.2021-21418

Wei M, Flowers L, Knight SAB, Zheng Q, Murga-Garrido S, Uberoi A, Pan JT-C, Walsh J, Schroeder E, Chu EW, Campbell A, Shin D, Bradley CW, Duran-Struuck R, Grice EA. Harnessing diversity and antagonism within the pig skin microbiota to identify novel mediators of colonization resistance to methicillin-resistant Staphylococcus aureus. mSphere 2023; https://doi.org/10.1128/msphere.00177-23

WHO. Global Antimicrobial Resistance and Use Surveillance System (GLASS). Geneva: World Health Organization; 2022.

Wieland M, Mann S, Guard CL, Nydam DV. The influence of 3 different navel dips on calf health, growth performance, and umbilical infection assessed by clinical and ultrasonographic examination. J Dairy Sci 2017; https://doi.org/10.3168/jds.2016-11654

Windeyer MC, Leslie KE, Godden SM, Hodgins DC, Lissemore KD, LeBlanc SJ. Factors associated with morbidity, mortality, and growth of dairy heifer calves up to 3 months of age. Prev Vet Med 2014; https://doi.org/10.1016/j.prevetmed.2013.10.019