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# BACTERIOLOGICAL AND CYTOLOGICAL FINDINGS IN POSTPARTUM DAIRY COWS WITH SUBCLINICAL ENDOMETRITIS

## CONSTATĂRI BACTERIOLOGICE ȘI CITOLOGICE LA VACILE DE LAPTE POSTPARTUM CU ENDOMETRITĂ SUBCLINICĂ

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**ABSTRACT**

Subclinical endometritis is a condition that produces negative effects on lactation yield and fertility of dairy cows during the puerperal period. The objective of the research consisted in the characterization of the uterine microbiota of dairy cows and the identification of pathogens associated with infertility. Differential selective media for aerobic and anaerobic bacteria were used for the isolation and identification of bacteria. To establish the diagnosis of subclinical endometritis, 54 animals were evaluated at ≥ 60 days postpartum; cytologically healthy cows (n = 32), cows with subclinical endometritis (n = 22). The microbiological examination was performed using samples of cervico-vaginal secretions collected from the same animals by vaginoscopy, namely 4 cows with endometritis and 4 healthy cows. Cervicovaginal secretions were examined microbiologically and the isolates identified included Gram-positive bacteria in percentage of 62.5% and 37.5% Gram-negative bacteria. The highest incidence was reported for *Escherichia coli* (16.66%), vancomycin-resistant *Enterococcus spp.* (16.66%), *Staphylococcus spp* (14.58%) and *Streptococcus agalactiae* (12.5%). The prevalence of subclinical endometritis, 40.7% (22/54) was established on the basis of cell ratios (≥ 5% polymorphonuclear cells) assessed following examination of endometrial smears. Antimicrobial susceptibility profile determination revealed high susceptibility to florfenicol (93.75%), enrofloxacin (91.66%), marbofloxacin (91.66%) followed by amoxicillin/clavulanic acid (83.33%) and cefquinome (70.83%). Antimicrobial resistance to tetracycline (100%), streptomycin (89.58%), colistin (72.91%) and penicillin (68.75%) was reported. In conclusion, the results of this study confirm that the combination of endometrial cytology with the microbiological examination of uterine discharges seems to be a good method for the diagnosis of subclinical endometritis, and provides an update of information about the microbiota present in the uterus of dairy cows after parturition, the state of its resistance to antibiotics and the effect on reproductive performance.

**Keywords**: dairy cows, subclinical endometritis, cytobrush technique, cervico-vaginal microbiota

**REZUMAT**

Endometrita subclinică este o afecțiune care produce efecte negative asupra randamentului lactației și fertilității vacilor de lapte în perioada puerperală. Obiectivul cercetării a constat în caracterizarea microbiotei uterine a vacilor de lapte și identificarea agenților patogeni asociați cu infertilitatea. Pentru izolarea și identificarea bacteriilor au fost utilizate medii selective diferențiale pentru bacterii aerobe și anaerobe. Pentru stabilirea diagnosticului de endometrită subclinică au fost evaluate 54 animale la ≥ 60 zile postpartum; vaci citologic sănătoase (n = 32), vaci cu SCE (n = 22). Examenul microbiologic a fost efectuat folosind probe de secreții cervico-vaginale colectate de la aceleași animale prin vaginoscopie, și anume 4 vaci cu endometrită și 4 vaci sănătoase. Secrețiile cervicovaginale au fost examinate microbiologic și izolatele identificate au inclus bacterii gram-pozitive în procent de 62,5% și 37,5% bacterii gram-negative. Cea mai ridicată incidență a fost semnalată la *Escherichia coli* (16,66%), *Enterococcus spp. vancomicino-rezistent* (16,66%), *Staphylococcus spp* (14,58%) și *Streptococcus agalactiae* (12,5%). Prevalența endometritei subclinice, 40,7% (22/54) a fost stabilită pe baza raporturilor celulare (≥ 5% celule polimorfonucleare) evaluate în urma examinării frotiurilor efectuate din endometru. Determinarea profilului de sensibilitate antimicrobiană a evidențiat o susceptibilitate ridicată față de florfenicol (93,75%) enrofloxacină (91,66%), marbofloxacină (91,66%) urmată de amoxicilină/acid clavulanic (83,33%) și cefchinomă (70,83%).A fost semnalată rezistență antimicrobiană față de tetraciclină (100%), streptomicină (89,58%), colistin (72,91%) și penicilină (68,75%). Rezultatele acestui studiu confirmă faptul că îmbinarea citologiei endometriale cu examenul microbiologic al secrețiilor uterine pare să fie o metodă bună de diagnostic al endometritei subclinice, și totodată oferă o actualizare a informațiilor despre microbiota prezentă în uterul bovinelor de lapte după parturiție, starea de rezistență a acesteia la antibiotice și efectul asupra performanței reproductive.

**Cuvinte cheie**: vaci de lapte, endometrită subclinică, tehnica cytobrush, microbiota cervico-vaginală

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Uterine conditions occurring after parturition have short- and long-term effects on the reproductive performance of dairy cattle (11). Endometritis is one of

the most important causes of infertility,repeated heats and increased culling rates in dairy cows (38, 45).

Clinical forms of endometritis are characterized by the appearance of muco-purulent or purulent vaginal discharge more than 21 days postpartum, with or without alteration of the general condition of the animal (40). Subclinical endometritis is defined by the absence of clinical signs and lack of vaginal discharge (18). Subclinical endometritis is also described as cytological endometritis and is characterized by the presence of a large number of polymorphonucleated cells in cytological samples collected from the endometrium by the cytobrush technique or uterine lavage (10). In dairy cows with subclinical endometritis, the percentage of PMN neutrophils has been observed to vary from ≥ 5% in samples collected at 40-60 days postpartum to > 18% when samples are collected from 20 days postpartum (40).

In the first two weeks after parturition, there is also an increased risk (80%) of bacterial contamination in dairy cows. Studies show that 25-40% of animals develop uterine inflammatory conditions in the first 21 days postpartum, which causes endometritis with clinical evolution in 15-20% of cases and subclinical evolution in about 30% (8, 21). The aetiology of endometritis is polymicrobial, being frequently involved both aerobic species of the genera *Escherichia, Bacillus, Enterococcus, Streptococcus,* and *Staphylococcus*, and anaerobic species of the genera *Fusobacterium, Proteus,* and *Prevotella* (5,47).

The main aim of the research was to identify the potentially pathogenic opportunistic microbiota in dairy cows with subclinical endometritis diagnosed by the cytobrush technique compared to clinically healthy cows and the influence of these microorganisms on reproductive performance.

## METHODS AND MATERIALS

The present study is part of a larger investigation involving 54 infertile multiparous Holstein cows that did not show signs of oestrus up to or after 60 days postpartum. The animals had no clinical signs of endometritis or other conditions generating the state of infertility. All cows came from the same herd, a cattle farm in N-E Romania, and were followed from calving until ≥ 60 days postpartum. Based on cytological samples collected from cows included in the study at ≥ 60 days after calving and in the absence of clinical signs of endometritis, cows were diagnosed with "subclinical endometritis" (SCE, n =22) or healthy (n =32). For eight retrospectively selected animals (healthy, n =4, SCE, n =4) the composition of the cervico-vaginal microbiota was also determined

Sample collection was performed after clinical examination of animals at ≥60 days after parturition. Cervico-vaginal discharge was collected after sanitizing the genital area and perineum with water and paper towels. Secretions were collected as aseptically as possible using sterile cotton swabs wrapped on 30 cm long metal rods, a method validated by other studies (27). Samples were collected individually by rolling the swab around the vaginal walls and cervical ostium. Transport of samples was done 2 hours after collection and under refrigerated conditions.

Bacteriological examination. The samples were processed in the Microbiology Laboratory of the Faculty of Veterinary Medicine, IULS "I. Ionescu de la Brad".

Direct smears-stained Gram and Ziehl-Neelsen (for acid-fast microorganisms) were carried out from the samples for general examination of microbial morphology. Following microscopic examination, samples were discharged into liquid medium for aerobic (Mueller Hinton broth, Oxoid) and anaerobic (anaerobic broth, Oxoid) bacteria. After incubation at 37°C for 24-48 hours under aerobic and anaerobic conditions, samples were transplanted onto various usual and special culture media used for bacterial isolation. Identification of type colonies was performed on Columbia + CNA + SheepBlood agar (BioRad) for isolation of Gram positive bacteria, Sa Select Medium (Bio Rad) for differentiation of *Staphylococcus species*, StrepB Select Agar (BioRad) for isolation of *Streptococcus agalactiae* species, group B streptococci, enterococci and lactobacilli, VRE Select Agar (Biorad) for isolation of *Enterococcus faecalis*, vancomycin resistant *Enterococccus faecium* species, Drigalsky Agar (Oxoid) for isolation of Gram negative bacteria, Chocolate PVS + Bacitracin (BioRad) for isolation of *Haemophilus species*, Schaedler + Vitamin K3 Agar (BioRad) for isolation of anaerobic bacteria. Isolation in pure cultures was performed on Muller-Hinton agar and blood agar to highlight haemolysis. Bacterial species were identified based on characteristics of isolated colonies, Gram staining, morphology, haemolysis, biochemical profile (API Systems) and other standard tests such as, serological identification of streptococcal strains using commercial latex particle agglutination, Prolex™ Strep kit (Pro-Lab Diagnostis) (6).

Susceptibility testing of bacterial strains to antibiotics was performed using microcompressors (BioRad): amoxicillin-clavulanic acid (AMC, 30 ug), cefquinome (CEQ 30 µg), colistin (COL,10 µg), enrofloxacin (ENR,5 µg), florfenicol (FFC,30 µg), gentamicin (GN, 30 µg), lincomycin (LCN,15 µg), marbofloxacin (MAR,5 µg), penicillin (P,10 µg), streptomycin (SMN, 10 µg), tetracycline (Te, 30 µg). The results of the antibiograms performed by the diffusimetric method, were interpreted according to the international standard EUCAST 2019 (European Committee on Antimicrobial Susceptibility Testing). Depending on the diameter of the zone of inhibition formed around the anti-

biotic tested, bacterial strains were classified as resistant, moderately susceptible or susceptible.

In order to identify cows with fertility problems, transrectal ultrasound examination of the uterus and cytological examination of the endometrial samples were performed to detect subclinical endometritis and to perform Metricure therapy 12h after artificial insemination (AI) in cows with endometritis.

For endometrial cytology, endometrial cell samples were collected by attaching a sterile swab to an artificial insemination pipette. The newly designed device was placed in a plastic protective sleeve to protect the cotton swab from vaginal contamination. In some cases, a vaginal speculum was used to minimise the risk of contamination.After successful penetration through the cervix up to the endometrium, using clockwise twisting movements, endometrial cells were harvested and absorbed by the cotton swab.

In the next step, the swab adapted to the AI pipette was carefully extracted and the endometrial cells were transferred on a microscopic slide by pressing the swab extracted from the uterus. After the cell smear was dried, it was deposited in a Petri dish and directed to the laboratory for staining and cytological examination. Staining was performed by the Diff-Quick method (Fisher Diagnostics, Newark, DE, USA) according to the manufacturer's instructions.

Determination of the percentage of PMN was performed by light microscopy (Leica DM 500), counting 10 fields of 10 cells each with x 400 objective (30).

The presence of uterine fluid in very small amounts, observable on transrectal ultrasound examination, without showing signs of chronic endometritis, was an additional criterion for including nonpregnant cows in the category of those with subclinical endometritis. In the case of diagnosing subclinical endometritis in infertile cows, two groups were made. A control group (MES group, n = 11) whose purpose was to evaluate by comparison the subclinical endometritis therapy applied to the experimental group (EES group, n = 11) but also to evaluate the healing capacity of this reproductive condition without therapy. In the case of the EES group, therapy applied was intrauterine administration of 500 mg Cephapirin benzathine (Metricure; Intervet, Whitby, Ont. Canada), 12 h after artificial insemination.

## RESULTS AND DISCUSSIONS

By associating the ultrasound diagnosis (to exclude other infertility-generating conditions) with the cytobrush technique (≥ 5% polymorphonuclear cells in the smear), infertile cows presented subclinical endometritis in a proportion of 40.7% (22/54).

Cattle in which values of less than 5% PMN were determined in the cytological smear were declared clinically healthy, while animals that had PMN >5% were diagnosed with subclinical endometritis (18, 42).

The cytological examination of the endometrial samples collected by cytobrush technique, numerous uniform endometrial epithelial cells and stromal cells were observed in healthy cows, and neutrophils and haematopoietic cells were present in low numbers. In cows that developed subclinical endometritis, a septic neutrophilic inflammation with numerous neutrophils and epithelial cells on a background of microorganisms was observed. Phagocytosed microorganisms and degenerated neutrophils embedded in mucus were present in smears from diseased cows. The use of the cytobrush method is considered to be a superior technique for collecting endometrial cells, including in humans and mares (19). The use of the cytobrush technique is preferred for cytological sampling of the endometrium as it is considered a non-invasive and less harmful technique compared to uterine lavage which, due to the saline fluid used, can cause irritation of the uterine lining (33).

Among the methods used for the diagnosis of subclinical endometritis the method that is considered to have the highest degree of confidence is the cytobrush method (3). Also, a single collection using the cytobrush technique yields both endometrial cells for cytological examination and pathological material represented by microorganisms, necessary for bacterial examinations and antibiograms (28). The collected secretions were bacteriologically analysed to identify conditionally pathogenic microbiota known to play a role in endometrial disease.

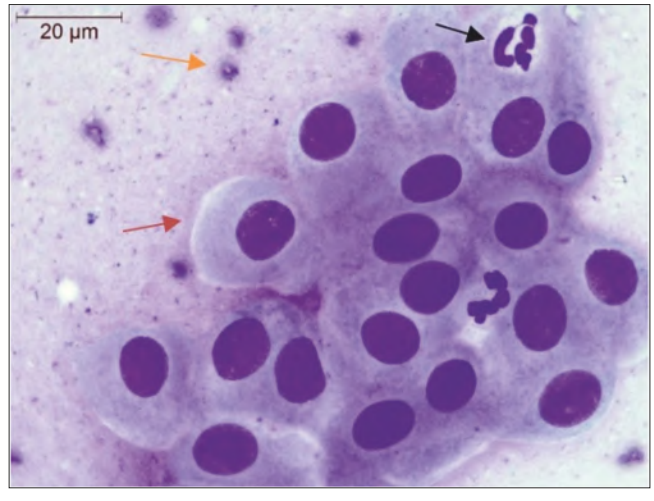


**Fig. 1.** Endometrial cytology of clinically healthy cow. The background is represented by mucus and sparse haematocytes (orange arrow). Numerous uniform epithelial cells (red arrow) and rare non-degenerate neutrophils (black arrow) are also present. Diff-Quick, x1000

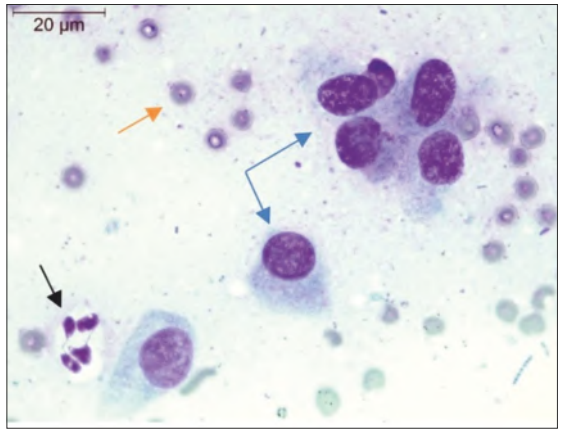


**Fig. 2.** Endometrial cytology of clinically healthy cow endometrium. Erythrocytes (orange arrow), stromal endometrial cells (blue arrow), and rare non-degenerate neutrophils (black arrow) can be noticed. Diff-Quick, x 1000

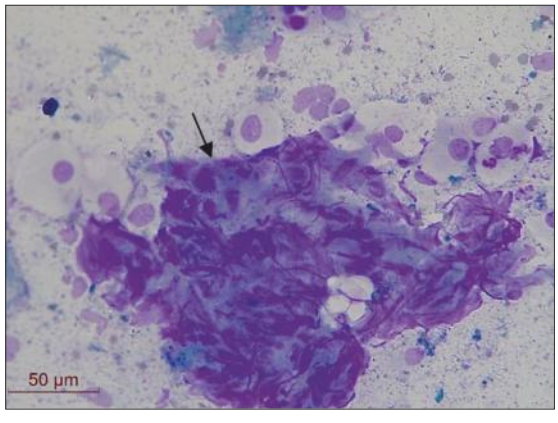


**Fig. 3.** Endometrial cytology in cows with subclinical endometritis. Cluster of degenerated epithelial cells are indicated by the black arrow. Diff-Quick, x400

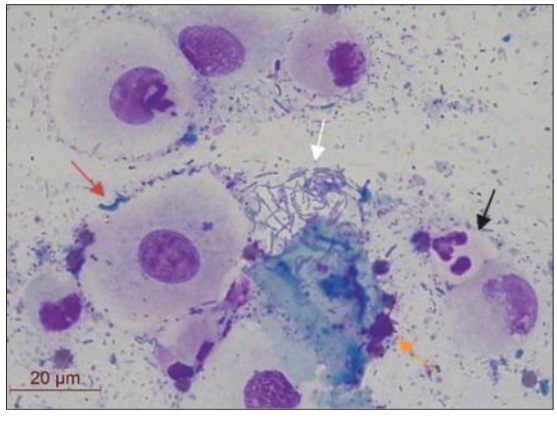


**Fig. 4**. Endometrial cytology in cows with subclinical endometritis. The background is represented by microorganisms (a group of bacilli is indicated by the white arrow) with numerous epithelial cells (red arrow). The erythocytes (orange arrow) and neutrophils (black arrow) are rare. Diff-Quick, x1000

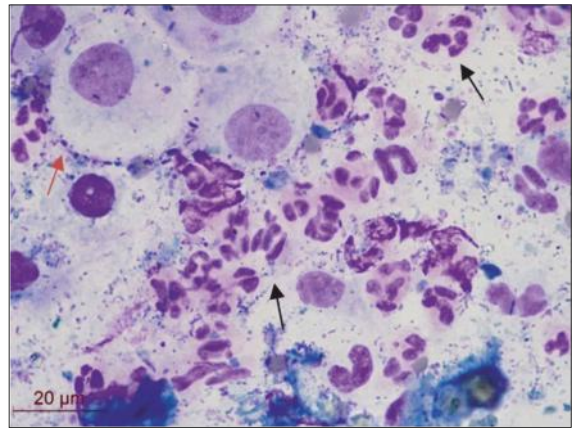


**Fig. 5.** Endometrial cytology in cow with subclinical endometritis. Septic neutrophilic inflammation represented by a background of microorganisms with numerous neutrophils (black arrow), some of them degenerated; and epithelial cells (red arrow). Diff-Quick, x1000

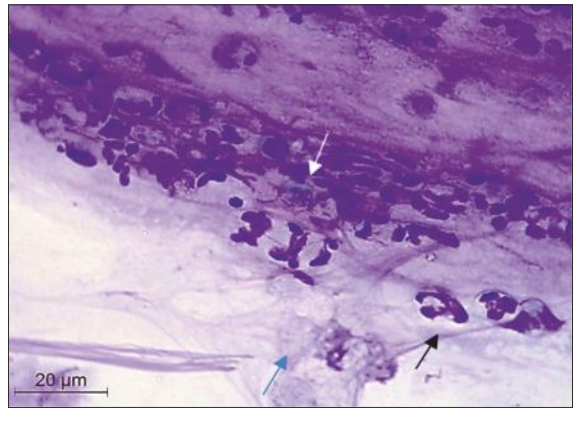


**Fig. 6.** Endometrial cytology in cow with subclinical endometritis. Septic neutrophilic inflammation: mucus blade (blue arrow) enclosing numerous degenerated neutrophils (black arrow) and phagocytosed microorganisms (white arrow). Diff-Quick, x 1000.

The uterine microbiota of clinically healthy cows and those with infertility problems were of the same microbial diversity. Microbiological examination of samples taken from 8 cows resulted in the isolation of 48 potentially pathogenic Gram-negative (37.5%) and Gram-positive (62.5%) bacterial strains.The most isolated microorganisms were classified in the genera: *Escherichia* (16.66%) and *Enterococcus (*16.66%),followed by *Staphylococcus* (14.58%), *Streptococcus* (12.5%), *Bacillus* (8.33%), *Proteus (*8.33%) and *Bacteroides* (6.25%) and *Clostridium* (6.25%) (Table 1).

Absence of altered vaginal discharge, presence of *Escherichia coli* species and induced neutrophil degeneration (50) can be considered predictive symptoms for subclinical endometritis.

**Table 1**

**Bacterial species isolated from cervico-vaginal secretions in cows with endometritis and susceptibility/resistance profile to antimicrobial agents tested**

| Bacterial species | | | No. of isolates | S/I/R | Antimicrobial agents tested | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | AMC | CEQ | COL | ENR | FFC | GMN | LCN | MAR | P | SMN | TET |
| | | | | | No.(%) | No.(%) | No.(%) | No.(%) | No.(%) | No.(%) | No.(%) | No.(%) | No.(%) | No.(%) | No.(%) |
| Gram positive | Aerobic | *Staphylococcus aureus* | 2 | S | 1 (50) | 1 (50) | 0 | 2 (100) | 2 (100) | 2 (100) | 1 (50) | 2 (100) | 1 (50) | 0 | 0 |
| | | | | I | 1 (50) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 (50) | 0 |
| | | | | R | 0 | 1 (50) | 2 (100) | 0 | 0 | 0 | 1 (50) | 0 | 1 (50) | 1 (50) | 2 (100) |
| | Aerobic | *Staphylococcus epidermidis* | 3 | S | 3 (100) | 0 | 0 | 3 (100) | 3 (100) | 3 (100) | 3 (100) | 3 (100) | 3 (100) | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 3 (100) | 3 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 3 (100) | 3 (100) |
| | Aerobic | *Staphylococcus saprophyticus* | 2 | S | 2 (100) | 2 (100) | 0 | 2 (100) | 2 (100) | 2 (100) | 2 (100) | 2 (100) | 2 (100) | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 (100) | 0 |
| | | | | R | 0 | 0 | 2 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 (100) |
| | Aerobic | *Streptococcus agalactiae* | 6 | S | 6 (100) | 2 (33.33) | 0 | 2 (33.33) | 4 (66.66) | 2 (33.33) | 0 | 2 (33.33) | 2 (33.33) | 0 | 0 |
| | | | | I | 0 | 2 (33.33) | 0 | 4 (66.66) | 2 (33.33) | 2 (33.33) | 2 (33.33) | 4 (66.66) | 0 | 2 (33.33) | 0 |
| | | | | R | 0 | 2 (33.33) | 6 (100) | 0 | 0 | 2 (33.33) | 4 (66.66) | 0 | 4 (66.66) | 4 (66.66) | 6 (100) |
| | Aerobic | *Enterococcus faecalis (VRE)* | 3 | S | 3 (100) | 3 (100) | 0 | 3 (100) | 3 (100) | 3 (100) | 0 | 3 (100) | 0 | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 0 | 3 (100) | 0 | 0 | 0 | 3(100) | 0 | 3 (100) | 3 (100) | 3 (100) |
| | Aerobic | *Enterococcus faecium(VRE)* | 2 | S | 2 (100) | 2 (100) | 0 | 2 (100) | 2 (100) | 2 (100) | 0 | 2 (100) | 0 | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 0 | 2 (100) | 0 | 0 | 0 | 2 (100) | 0 | 2 (100) | 2 (100) | 2 (100) |
| | Aerobic | *Enterococcus gallinarum* | 3 | S | 3 (100) | 3 (100) | 0 | 3 (100) | 3 (100) | 3 (100) | 0 | 3 (100) | 0 | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 0 | 3 (100) | 0 | 0 | 0 | 3 (100) | 0 | 3 (100) | 3 (100) | 3 (100) |
| | Aerobic | *Corynebacterium spp.* | 2 | S | 2 (100) | 2 (100) | 0 | 2 (100) | 2 (100) | 2 (100) | 0 | 2 (100) | 2 (100) | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | | 0 | 2 (100) | 0 | 0 | 0 | 2 (100) | 0 | 0 | 2 (100) | 2 (100) |
| | Aerobic | *Bacillus cereus* | 4 | S | 4 (100) | 0 | 0 | 4 (100) | 4 (100) | 0 | 0 | 4 (100) | 0 | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 4 (100) | 0 | 0 | 4 (100) | 0 | 0 |
| | | | | R | 0 | 4 (100) | 4 (100) | 0 | 0 | 0 | 4 (100) | 0 | 0 | 4 (100) | 4 (100) |
| | Anaerobic | *Clostridium perfringens* | 3 | S | 1 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 0 | 0 | 1 (100) | 0 | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 0 | 1 (100) | 0 | 0 | 1 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) |
| Gram negative | Aerobic | *Escherichia coli* | 8 | S | 8 (100) | 8 (100) | 0 | 8 (100) | 8 (100) | 0 | 0 | 8 (100) | 0 | 0 | 0 |
| | | | | I | 0 | 0 | 8 (100) | 0 | 0 | 8 (100) | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 0 | 0 | 0 | 0 | 0 | 8 (100) | 0 | 8 (100) | 8 (100) | 8 (100) |
| | Aerobic | *Klebsiella spp.* | 1 | S | 1 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 0 | 1 (100) | 0 | 0 | 0 | 1 (100) | 0 | 0 | 1 (100) | 1 (100) |
| | Aerobic | *Proteus vulgaris* | 4 | S | 0 | 4 (100) | 0 | 4 (100) | 4 (100) | 0 | 0 | 4 (100) | 0 | 0 | 0 |
| | | | | I | 4 (100) | 0 | 0 | 0 | 0 | 4 (100) | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 0 | 4 (100) | 0 | 0 | 0 | 4 (100) | 0 | 4 (100) | 4 (100) | 4 (100) |
| | Anaerobic | *Bacteroides spp.* | 3 | S | 0 | 3 (100) | 0 | 3 (100) | 3 (100) | 0 | 0 | 3 (100) | 0 | 0 | 0 |
| | | | | I | 3 (100) | 0 | 0 | 0 | 0 | 3 (100) | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 0 | 3 (100) | 0 | 0 | 0 | 3 (100) | 0 | 3 (100) | 3 (100) | 3 (100) |
| | Anaerobic | *Fusobacterium necrophorum* | 1 | S | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) | 0 | 0 | 1 (100) | 0 | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 1 (100) | 0 | 0 | 0 | 1 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) |
| | Anaerobic | *Prevotella spp.* | 1 | S | 1 (100) | 0 | 1 (100) | 1 (100) | 0 | 0 | 0 | 1 (100) | 0 | 0 | 0 |
| | | | | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | R | 0 | 1 (100) | 0 | 0 | 1 (100) | 1 (100) | 1 (100) | 0 | 1 (100) | 1 (100) | 1 (100) |

Legendă/Legend: AMC-Amoxycillin/Clavulanic Acid; CEQ-Cefquinome; COL-Colistin; ENR-Enrofloxacin; FFC-Florfenicol; GMN- Gentamicin; LCN - Lincomycin; MAR - Marbofloxacin; P - Penicillin, SMN - Streptomycin; TET- Tetracycline, S – Susceptibil, I – Intermediar, R – Rezistent

Studies show that *Escherichia coli* associated with strictly anaerobic Gram-negative pathogenic species such as *Fusobacterium necrophorum* and *Bacteroides spp*. are responsible for uterine disorders evolving in dairy cows (4, 32, 39).

*Clostridium perfringens* and species of the genus *Prevotella* participate significantly in the pathogenesis of endometritis (9). Also, aerobic Gram-positive species belonging to the genera *Bacillus*, *Staphylococcus*, *Streptococcus* and *Enterococcus* are frequently associated in the evolution of uterine disorders in dairy cows (36). Treatment of bacterial infections has become a challenge due to the ability of bacteria to develop resistance to antimicrobial agents. The emergence of multidrug-resistant (MDR) germs is observed globally and is a problem for animal and public health because of their ability to spread to humans (44).

Worldwide, the most common antibiotics used in the therapies of uterine disorders in dairy cows are oxytetracycline, enrofloxacin, penicillin, streptomycin, cefquinome, and cephalosporins (43).

In our study, to determine the susceptibility profile of bacterial strains isolated in pure or mixed cultures, 11 antibiotics, commonly used in current practice for the treatment of endometritis in cows, were tested (Table 1). Synthetic analysis of the data obtained showed that the Gram-negative species isolated expressed a multi drug resistant (MDR) phenotype to several antimicrobial agents from different classes of antibiotics such as colistin (polymyxins), lincomycin (lincosamides), penicillin (penicillins), streptomycin (aminoglycosides) and tetracycline (tetracyclines). In regard to penicillin and tetracycline being the mainstay antibiotics in the treatment of uterine infections, the resistance encountered in the cows studied indicates the transmission of a mechanism that led to increased microbial resistance (49).

Tetracyclines act on bacteria by reversibly inhibiting the 30S ribosomal subunit, and when resistance sets in, the mechanisms are unclear but it appears that efflux pumps and ribosomal protection are affected (16). In terms of penicillin resistance, Gram-positive and Gram-negative bacteria respond differently due to structural differences, as Gram-negatives have an outer membrane that blocks penicillin penetration differently and secondly, they can synthesize specific genes encoding penicillinases (beta-lactamases) responsible for inactivating penicillin by hydrolysis of the beta-lactam ring (22).

*Escherichia coli* strains showed 100% (8/8) resistance to tetracycline, lincomycin, penicillin and streptomycin and moderate (100%) susceptibility to colistin and gentamicin.

Colistin is considered the antibiotic of choice for human infections caused by multidrug-resistant *Enterobacteriaceae*, especially ESBL-producing *Enterobacteriaceae*. Polymyxins are active mainly against Gram-negative bacilli such as *Escherichia coli* and *Klebsiella spp*., but not against Gram-positive bacteria, Gram-negative cocci and anaerobes. Resistance of some *Enterobacteriaceae* to colistin can be intrinsic as in the case of *Proteus spp.* or mediated by structural changes in both the cytosol and cell membrane in *Klebsiella spp*. and *Escherichia coli* (7).

According to Malinowski (2010), due to antimicrobial efficacy, fluoroquinolones (enrofloxacin and florfenicol) should be considered in first-line treatment for endometritis produced by Gram-negative aerobic germs such as *Escherichia coli, Klebsiella spp*. and *Proteus spp*. It also identified a high susceptibility of the anaerobic species, *Bacteroides spp.,* to fluoroquinolones. Similar results were also obtained in our study, with fluoroquinolones being the antimicrobial agents with the highest efficacy (93.75%).

Amoxicillin / Clavulanic acid is also highly active against anaerobic bacteria such as *Bacteroides spp*., *Prevotella spp*. and *Fusobacterium necrophorum* (25). Thus, in terms of susceptibility to Amoxicillin / Clavulanic acid, we observed a high susceptibility (100%) of Gram-negative bacterial species represented by *Escherichia coli*, *Fusobacterium necrophorum*, *Klebsiella spp*., and *Prevotella spp*.

Increased resistance to penicillin (53.33%), lincomycin (73.33%), streptomycin (83.33%) and tetracycline (100%) was reported for Gram-positive strains isolated from study animals.

Species of the genus *Bacillus* are considered opportunistic and ubiquitous uterine pathogens in the environment, feed and milk of cows farmed for dairy production (48).

Surprisingly, *Bacillus cereus* species isolated in our study showed 100% resistance to cefquinome, lincomycin, streptomycin and tetracycline. The phenomenon of multi-drug resistance of isolated *Bacillus cereus* strains was also present in other studies of resistance to chloramphenicol-glycopeptide-tetracycline-cephalosporin-β-lactams. This resistance is natural for lincomycin due to resistance genes to this antimicrobial agent, genes that have been described as part of the genome in the genus *Bacillus* and are considered intrinsic (1). *Bacillus cereus* is usually resistant to β-lactam antibiotics due to the production of β-lactamases (29). Moreover, it is able to acquire resistance to antimicrobial agents commonly used in therapy, such as tetracycline, which is mostly used intrauterine and/or intramuscular administration, and streptomycin (43).

*Clostridium perfringens* isolates showed 100% resistance to colistin, gentamicin, penicillin, lincomycin, streptomycin and tetracycline.

*Staphylococcus* strains were classified as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus*.Of these,100% (7/7) were

resistant to colistin and tetracycline, 57.1% (4/7) were resistant to cefquinome and streptomycin and 14.28% (1/7) were resistant to lincomycin and penicillin. Tetracycline and penicillin resistance genes have been found in staphylococci and are indicative of methicillin resistance (51). In our study, no methicillin-resistant strains of *Staphylococcus aureus* were isolated (MRSA). *Streptococcus agalactiae* isolates showed resistance to antimicrobial agents used in the therapy of infections caused by them, such as: penicillin 66.66% (4/6), tetracycline 100% (6/6), lincomycin 66.66% (4/6) and streptomycin 66.66% (4/6).

In contrast, 100% susceptibility was reported to amoxicillin /clavulanic acid, streptococci being major pathogens unable to acquire exogenous beta-lactam resistance genes, an observation also reported by other studies (23). An intermediate susceptibility of group B streptococci was observed for the antimicrobial agents of the fluoroquinolone class, enrofloxacin (66.66%) and marbofloxacin (66.66%).

Antibiotics belonging to the quinolone class are not active against streptococci because of their intrinsic resistance. However, fluoroquinolones may be an alternative to beta-lactam antibiotics for treating streptococcal infections (14).

Two beta-haemolytic *Corynebacterium spp*. strains isolated from mixed cultures of staphylococci and streptococci have also been identified.

Group D streptococci were represented by *Enterococcus faecalis* 37.5% (3/8), *Enterococcus gallinarum* 37.5% (3/8) and *Enterococcus faecium* 25% (2/8). Enterococci expressed a multi drug resistant (MDR) phenotype to several antimicrobial agents. They showed 100% resistance to colistin (polymyxins), lincomycin (lincosamides), penicillin (penicillins), streptomycin (aminoglycosides) and tetracycline (tetracyclines). These trends have also been reported in previous studies, where *Enterococcus faecium* resistance to tetracycline was 45% (26).

Studies have reported human and animal clinical isolates of *Enterococcus* showing resistance to antimicrobial agents such as macrolides, tetracyclines, streptogramins and glycopeptides. Currently, vancomycin-resistant enterococci (VRE) have become a major therapeutic challenge due to intrinsic resistance and the potential to develop resistance to almost all classes of antibiotics of importance to human medicine, such as cephalosporins, beta-lactams and aminoglycosides (15).

Following antibiogram evaluation, it can be seen that there are increased levels of resistance to the antibiotics tested and evidence of developing resistance to important human antimicrobials such as penicillins, aminoglycosides and tetracyclines. This is either due to the application of a broad spectrum of antimicrobial agents in dairy cattle therapy leading to the formation of multidrug-resistant microflora ("superbugs") or to interspecies cross transfer. These superbugs can travel through the food chain to humans and become sources of antibiotic resistance genes for pathogens in clinical infections (20).

The correlation of microbiological examination results with cytological examination, which allowed the determination of the percentage of inflammatory cells ranging from 7-30% PMN at ≥ 60 days postpartum, constitutes a real and permissive model for establishing the diagnosis of subclinical endometritis, a point also supported by other authors (10, 18, 41). According to Bajaj (2016) endometrial cytology performed by cytobrush technique is the most effective diagnostic method for subclinical endometritis when used in conjunction with uterine microbiota screening.

Regarding the prevalence of subclinical endometritis, our results are consistent with other studies. Thus, Gilbert (2005) and Senosy (2012) reported the disease in 53% of the herd at 40-60 days postpartum and 52% at 46 days, respectively. The high prevalence of subclinical endometritis in dairy cattle is always associated with infertility, increased pregnancy rate, delayed first service and substantially decreased first service pregnancy rate.

It should be noted that the cytological diagnosis, in addition to an increased number of polymorphonuclear cells, often shows a degree of uterine hyperplasia and numerous extracellular bacteria, which may have influenced the rate of conception.

Transrectal ultrasonographic evaluation in combination with uterine cytological examination in this study revealed that a large proportion of this degree of infertility is caused by the presence of an increased proportion of polymorphonuclear cells in the uterine slides (≥ 5% of the number of cells identified) in association with uterine hyperplasia and numerous extracellular bacteria. We mention that this percentage may vary from study to study and depending on the minimum polymorphonuclear cell threshold used in the diagnosis. Application of Metricure therapy 12 hours after artificial insemination to improve reproductive performance did not result in a significant increase in conception rate in the EES group compared to the MES group (36.4% vs. 27.3%; $P > 0.05$). Similarly, Galvao (2009) demonstrated that intrauterine administration of Ceftiofur did not significantly improve conception rates in cows diagnosed with endometriosis but this treatment has the potential to reduce the prevalence of intrauterine infections.

## CONCLUSIONS

Our results showed that after 60 days postpartum, in dairy cows a cervico-vaginal microbiota develops that includes a multitude of pathogenic or opportunis-

tic bacterial species classified in the genera *Streptococcus*, *Staphylococcus*, *Escherichia*, *Clostridium*, *Fusobacterium*, *Bacillus*, *Corynebacterium*, *Prevotella* and *Bacteroides,* which increasing the risk of infertility.

This study demonstrates that the use of transrectal ultrasonography to exclude other reproductive disorders in combination with assessment of the percentage of polymorphonuclear cells in the uterine smear and microbiological examination can be used to establish the diagnosis of subclinical endometritis as a causative factor of infertility in dairy cows. It is possible that uterine hyperplasia associated with increased polymorphonuclear cell counts observed in cows in this category may influence implantation and normal gestational development.

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