RENAN BRAGA PAIANO

Endometritis in dairy cows reared in tropical conditions: microorganisms, reproductive performance and natural alternative therapy

São Paulo

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Endometritis in dairy cows reared in tropical conditions: microorganisms, risk factors, reproductive performance and natural alternative therapy

Thesis submitted to the Postgraduate Program in Animal Reproduction of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Doctor's degree in Sciences.

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Advisor:

Prof. Dr. Pietro Sampaio Baruseli

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Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

CERTIFICADO

Certificamos que a proposta intitulada "Endometrite em vacas leiteiras criadas em condições tropicais: microrganismos, fatores de risco, desempenho reprodutivo e terapia alternativa natural", protocolada sob o CEUA nº 1377270218 (ID 006772), sob a responsabilidade de **Pietro Sampaio Baruselli** *e equipe; Renan Braga Paiano* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 02/07/2019.

We certify that the proposal "Endometritis in dairy cows reared in tropical conditions: Microorganisms, reproductive performance and natural alternative therapy", utilizing 399 Bovines (399 females), protocol number CEUA 1377270218 (ID 006772), under the responsibility of **Pietro Sampaio Baruselli** *and team; Renan Braga Paiano* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 07/02/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 06/2019 a 10/2021 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	2 a 10 anos	N:	399
Linhagem:	Holandês Preto e Branco			Peso:	400 a 890 kg		

Local do experimento: USP-PIRASSUNUNGA, Fazenda Agrindus e Fazenda da Força Aérea Brasileira

São Paulo, 09 de novembro de 2021

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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EVALUATION FORM

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I dedicate this thesis to my family who have always been the source of inspiration in my life, especially to my grandfather who always encouraged me to take care of animals.

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RESUMO

PAIANO, R.P. **Endometrite em vacas leiteiras criadas em condições tropicais:** microrganismos, fatores de risco, desempenho reprodutivo e terapia alternativa natural. 2021. 120 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

Doenças uterinas causam redução da lucratividade das granjas leiteiras além de prejudicar a fertilidade das vacas. No entanto, faltam informações detalhadas sobre a eficiência reprodutiva e produtiva de vacas leiteiras afetadas por doenças uterinas em condições tropicais, além da identificação dos fatores e dos microrganismos associados com a ocorrência dessa enfermidade. Destaca-se, também, a importância de estudos relacionados aos possíveis tratamentos naturais e não convencionais para doenças uterinas em substituição à antibioticoterapia. Dessa forma, essa tese foi estruturada em três estudos: 1) Identificar os principais microrganismos presentes no ambiente uterino de vacas leiteiras com doenças uterinas durante o puerpério; 2) Identificar os principais fatores associados à ocorrência de doenças uterinas e avaliar o seu impacto no desempenho reprodutivo e na produção de leite. 3) Avaliar a ação antibacteriana *in vitro* pelo teste de difusão em disco de sete óleos essenciais (alecrim, canela, cravo, eucalipto, limão, orégano e tomilho) contra as cepas padrão de *Escherichia coli* (ATCC 25922), *Fusobacterium necrophorum* (ATCC 25286) e *Trueperella pyogenes* (ATCC 19411). No estudo 1, o objetivo foi avaliar os principais microrganismos relacionados aos casos de endometrite clínica (EC) e endometrite subclínica (ES) em amostras uterinas de vacas leiteiras em rebanhos brasileiros. Foram utilizados meios seletivos e diferenciais para o isolamento de bactérias aeróbicas e anaeróbicas e posterior identificação por espectrometria de massa (MALDI-TOF MS). Um total de 279 vacas Holandesas em lactação com 28 a 33 dias em leite de seis fazendas comerciais foi avaliado. Inicialmente, as vacas foram classificadas em três grupos: vacas saudáveis (n = 161), vacas com EC (n = 83) e vacas com ES (n = 35). Um total de 127 espécies bacterianas de 48 gêneros foi identificado. Animais saudáveis apresentaram 97 espécies, seguidos do grupo EC com 53 espécies, enquanto vacas ES apresentaram apenas 21 espécies bacterianas. Houve uma taxa de isolamento significativamente maior de *Trueperella pyogenes* em vacas com EC (26,5%) em comparação com vacas saudáveis e ES. Algumas espécies anaeróbias foram isoladas exclusivamente do grupo EC. Curiosamente, 18,1% das amostras de vacas EC e 40% das vacas ES foram negativas ao isolamento bacteriano. No estudo 2, o objetivo foi investigar os impactos da endometrite clínica e da endometrite subclínica no desempenho reprodutivo e na produção de leite de vacas criadas em condições tropicais. Um total de 279 vacas Holandesas

em lactação (28 a 33 dias em leite) de seis fazendas comerciais foram estudadas. Os animais foram classificados em três grupos: vacas saudáveis (sem EC e ES, n = 161), vacas com EC (escore de corrimento vaginal = 3 e ≥ 18% PMNL, n = 83) e vacas com ES (ausência de sinais de EC e > 18% PMNL, n = 35). Vacas com EC apresentaram menor taxa de concepção à primeira IA (P < 0,05), e ambas as vacas com EC e ES necessitaram maior número de serviços e de dias para emprenhar (P < 0,05), além de produzirem menos leite do que vacas saudáveis (P < 0,05). Nenhum fator avaliado neste estudo foi associado à ocorrência de EC e ES (P > 0,05). Em conclusão, foi evidenciado impacto negativo de EC e ES no desempenho reprodutivo e na produção de leite de vacas leiteiras. No estudo 3, foi investigada a atividade antibacteriana de sete óleos essenciais contra as cepas de referência de *Escherichia coli*, *Fusobacterium necrophorum*, *Trueperella pyogenes* e *Staphylococcus aureus*. O ensaio de difusão em disco revelou que os óleos essenciais de canela, cravo, orégano e tomilho apresentaram maior zona de inibição contra todas as bactérias avaliadas. Esses achados indicam que os óleos essenciais apresentam potencial para serem utilizados como alternativa no tratamento da endometrite bovina. No geral, os achados da presente tese revelaram quais são os principais microrganismos presentes no ambiente uterino de vacas leiterias com e sem endometrite criadas em condições tropicais. Verificou-se, ainda, que a endometrite clínica e subclínica prejudicaram a performance reprodutiva e a produção de leite. Por fim, os óleos essenciais apresentam forte atividade antibacteriana diante dos principais microrganismos associados com doenças uterinas em vacas leiteiras.

Palavras-chave: Endometrite clínica. Endometrite subclínica. Microrganismos. Performance reprodutiva. Vacas leiteiras.

ABSTRACT

PAIANO, R.P. **Endometritis in dairy cows reared in tropical conditions:** microorganisms, risk factors, reproductive performance and natural alternative therapy. 2021. 120 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2021.

Uterine diseases reduce the profitability of dairy farms in addition to impairing the fertility of dairy cows. However, there is a lack of detailed information on the reproductive and productive performance of dairy cows affected by uterine diseases in tropical conditions, in addition to identification of factors and microorganisms associated with the occurrence of this disease. The importance of studies related to possible natural and unconventional treatments for uterine in replacement of antibiotic therapy is also highlighted. Thus, this thesis was structured in three studies: 1) Identify the main microorganisms present in the uterine environment of dairy cows with uterine diseases during the puerperium; 2) Identify the main factors associated with the occurrence of uterine diseases in dairy cows and assess the impact their impact on reproductive performance and milk production. 3) Evaluate the *in vitro* antibacterial action by disk diffusion assay of seven essential oils (rosemary, cinnamon, clove, eucalyptus, lemon, oregano and thyme) against the reference strains of *Escherichia coli* (ATCC 25922), *Fusobacterium necrophorum* (ATCC 25286) and *Trueperella pyogenes* (ATCC 19411). In study 1, the objective was to assess the main microorganisms related to cases of clinical endometritis (CE) and subclinical endometritis (SE) from uterine samples of dairy cows in Brazilian herds. Selective and differential media were used for isolation of aerobic and anaerobic bacteria and subsequent identification by mass spectrometry (MALDI-TOF MS). A total of 279 lactating Holstein cows with 28 to 33 days in milk from six commercial farms were evaluated. Initially, cows were classified in three groups: cytologic healthy cows (n = 161), cows with CE (n = 83), and cows with SE (n = 35). A total of 127 bacterial species of 48 genera were identified. Healthy animals presented 97 species, followed by the CE group with 53 identified species, while SE cows had only 21 bacterial species. There was a significantly higher isolation rate of *Trueperella pyogenes* in CE cows (26.5%) compared to healthy and SE cows. Some anaerobic species were exclusively isolated from the CE group. Interestingly, 18.1% of samples from CE cows and 40% of SE cows were negative to bacterial isolation. In study 2, the objective was to investigate the impacts of clinical endometritis and subclinical endometritis on the reproductive performance and milk production of cows reared in tropical conditions. A total of 279 lactating Holstein dairy cows (28 to 33 d in milk) from six commercial farms were studied. The animals

were classified into three groups: healthy cows (without CE and SE, n = 161), cows with CE (vaginal discharge score = 3 and ≥ 18% PMNL, n = 83) and cows with SE (absence of signs of CE and > 18% PMNL, n = 35). Cows with CE had a lower conception rate at first AI ($P < 0.05$), and both cows with CE and SE required more services and days to be pregnant ($P < 0.05$), in addition to producing less milk than healthy cows ($P < 0.05$). No factor evaluated in this study was associated with the occurrence of CE and SE ($P < 0.05$). In conclusion, a negative impact of CE and SE on the reproductive performance and milk production of dairy cows was evidenced. In study 3, the antibacterial activity of seven essential oils against the reference strains of *Escherichia coli*, *Fusobacterium necrophorum*, *Trueperella pyogenes* and *Staphylococcus aureus* was investigated. The disk diffusion test revealed that the essential oils of cinnamon, clove, oregano and thyme showed presented a greater zone of inhibition against all bacteria evaluated. These findings indicate that essential oils have potential to be used as an alternative in the treatment of bovine endometritis. Overall, the findings of this thesis reveal the main microorganisms present in the uterine environment of cows with and without endometritis raised in tropical conditions. It was also found that clinical and subclinical endometritis impaired reproductive performance and milk production. Finally, essential oils have a strong antibacterial activity against the main microorganisms associated with uterine diseases in dairy cows.

Keywords: Clinical endometritis. Subclinical endometritis. Microorganisms. Reproductive performance. Dairy cows.

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SUMMARY

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

Agribusiness is responsible for approximately ¼ of the Brazilian Gross Domestic Product (GDP), generating an estimated GDP of around R$ 1.5 trillion (BRASIL, 2020). The milk production chain is one of the main economic activities in Brazil, with a strong effect on the generation of jobs and income, employing more than one million people in the countryside (BRASIL, 2020). In 2019, the gross value of primary milk production in Brazil reached almost R$ 35 billion, the seventh largest among national agricultural products (BRASIL, 2020). Brazil occupies a prominent position in relation to the milk producing countries. According to FAO, (2019), Brazil ranks fourth in the ranking of the largest milk producing countries in the world, with a production of 35.17 million tonnes in 2019, surpassed only by Pakistan (47.30 million tonnes), the United States (99.16 million tonnes) and India (196.18 million tonnes). The total number of cows milked in 2019 was 16.3 million, 0.5% lower compared to 2018 (BRASIL, 2020). Milk productivity corresponds to 2,147 liters per cow per year, however, each animal contributes only 5.88 liters per day, evidencing the low productivity (BRASIL, 2020).

The main causes of low national productivity are the lack of adaptation of dairy cows to climatic conditions that can accentuate heat stress. It has been reported that genetic factors, lack of adequate facilities that harm animal welfare, lack of qualified labor, in addition to diseases of animals can influence the efficiency of milk production (PAIANO, 2018; PAIANO et al., 2018; BARUSELLI et al., 2019; D'OCCHIO et al., 2019; PAIANO et al., 2019a; BARUSELLI et al., 2020). Furthermore, the uterine diseases contribute to a marked economic loss in dairy farms (FIGUEIREDO et al., 2021; MERENDA et al., 2021; PAIANO et al., 2021).

Uterine diseases can affect milk production on farms, reducing producers' profitability (PAIANO et al., 2019b; PAIANO et al., 2019c; FIGUEIREDO et al., 2020; PAIANO et al., 2020a; PAIANO et al., 2020b; PINEDO et al., 2020; YASUOKA et al., 2020; PÉREZ-BÁEZ et al., 2021; SILVA et al., 2021). The main uterine diseases that cause reduced reproductive performance and negatively impact farm productivity are metritis and endometritis.

Due to the lack of information about the impact of the uterine diseases on reproduction and milk production in Brazilian herds, new studies are needed to contribute to the measures of decisions on farms on the forms of prevention, diagnosis and treatments.

2 LITERATURE REVIEW

2.1 Uterine diseases

Metritis is a disease that is diagnosed within the first 3 weeks after parturition, and can be classified into puerperal metritis and clinical metritis (SHELDON et al., 2006) (Table 1). Puerperal metritis is characterized by an abnormally enlarged uterus with a fetid red-brown uterine discharge, which is associated with signs of systemic disease, including anorexia, depression, decreased milk yield and feed intake and fever (≥39.5°C) (SHELDON et al., 2006). Clinical metritis is characterized by an abnormally enlarged uterus, with the presence of purulent uterine discharge with no sign of systemic disease and fever (SHELDON et al., 2006). The incidence of metritis can be greater than 25% depending on the hygiene conditions in which the cows are housed during the puerperium (LEBLANC, 2008; GALVÃO, 2012; PAIANO et al., 2020c).

Endometritis is characterized as a superficial inflammation of the endometrium (GILBERT et al., 2005) (Table 1). Recently, endometritis has been subdivided into clinical endometritis (CE) and subclinical endometritis (SE) (DRILLICH AND WAGENER, 2018). Clinical endometritis, also known as purulent vaginal discharge (DUBUC et al., 2010), is a disease characterized by the presence of purulent vaginal discharge 21 days after parturition or mucopurulent discharge 26 days after parturition (SHELDON et al., 2006). The incidence of CE reported in previous studies can exceed the rate of 30% of affected cows (PLÖNTZKE et al., 2011; GONZALEZ-PENA et al., 2016).

Subclinical endometritis, also known as cytological endometritis (DUBUC et al., 2010), is a condition characterized by inflammation of the endometrium, without the presence of purulent or mucopurulent vaginal discharge, being diagnosed according to the presence of polymorphonuclear leukocytes (PMNL) in uterine cytology (KASIMANICKAM et al., 2004) (Table 1). The main cutoff points used in the diagnosis of SE are > 18% PMNL in uterine samples taken between 21 and 33 after calving, or > 10% PMNL in uterine samples taken between 34 and 47 after delivery (KASIMANICKAM et al., 2004; SHELDON et al., 2006). Subclinical endometritis can affect more than 70% of postpartum dairy cows (BARANSKI et al., 2012; GILBERT et al., 2005), however the incidence of SE can vary between farms according to the cutoff point of PMNL used and the postpartum period when samples are taken (WAGENER et al., 2017).

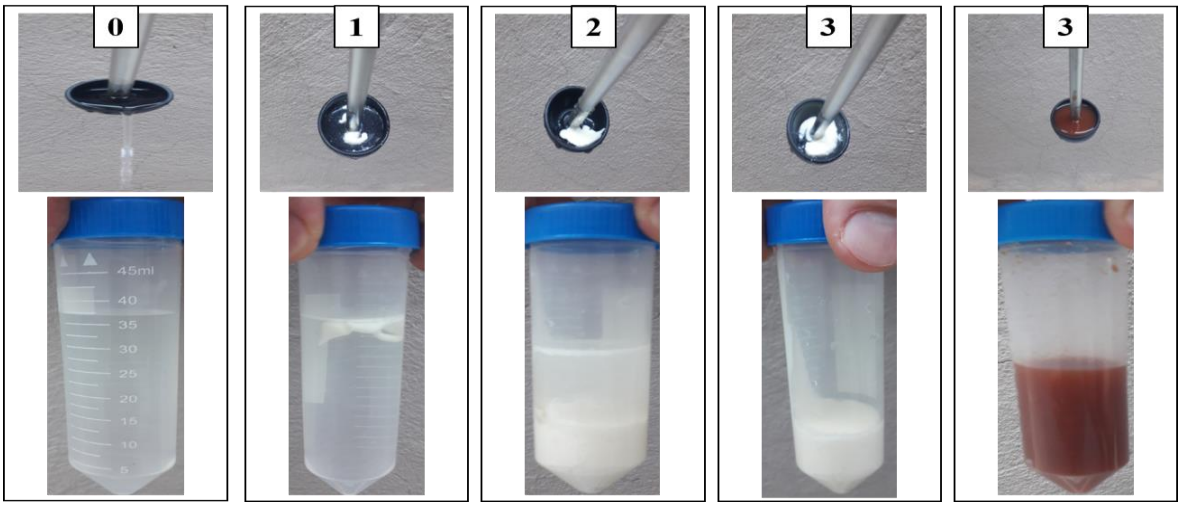
Table 1. Definition and diagnosis of the main uterine diseases of dairy cows

Disease	**Definition**	**Diagnosis**
Puerperal metritis	Abnormally enlarged uterus with a fetid red-brown uterine discharge, presence of signs of systemic illness and fever (≥39.5°C) within 21 days postpartum.	Inspection of vaginal discharge (transrectal/vaginal palpation, transrectal ultrasound, metricheck device).
Clinical metritis	Abnormally enlarged uterus, with the presence of purulent uterine discharge with no sign of systemic disease and fever within 21 days postpartum.	Inspection of vaginal discharge (transrectal/vaginal palpation, transrectal ultrasound, metricheck device).
Clinical endometritis	Purulent or mucopurulent uterine discharge 21 days or more postpartum.	Inspection of vaginal discharge (transrectal/vaginal palpation, transrectal ultrasound, metricheck device, vaginoscopy).
Subclinical endometritis	Uterine inflammation in the absence of purulent or mucopurulent uterine discharge 21 days or more postpartum.	Measuring the proportion of PMNL in uterine cytology.

The main risk factors associated with uterine diseases include body condition score, immunodepression, metabolic diseases, dystocia, male offspring, stillbirth, abortions, twins, cesarean section and placenta retention, among others (ADNANE et al., 2017; CHEONG et al., 2011; POTTER et al., 2010).

With regard to diagnosis, metritis and clinical endometritis can be diagnosed by assessing vaginal discharges, with the gloved hand techniques, vaginoscopy and metricheck tool (LEBLANC et al., 2002; WILLIAMS et al., 2005; MCDOUGALL et al., 2007). In addition to the evaluation of the endometrium by ultrasound (PAIANO et al., 2019d). The vaginal mucus score for uterine diseases (Figure 1) can be classified as: 0 = clear or translucent mucus, 1 = mucus containing streaks of pus, 2 = mucus containing ≤ 50% pus, 3 = mucus containing > 50% pus, or reddish-brownish watery fetid discharge.

Figure 1. Vaginal discharge scoring system for postpartum dairy cows.



(0) Clear discharge; (1) discharge with speck of pus; (2) Mucopurulent discharge; (3) Purulent discharge; (3) Reddish-brownish watery fetid discharge.

Fonte: Paiano, R. B. (2021).

The diagnosis of subclinical endometritis is characterized by an increase in the proportion of PMNL in uterine samples collected by uterine cytology (SHELDON et al., 2006). The main techniques described to perform endometrial cytology are cytobrush (KASIMANICKAM et al., 2004) and cytotape (PASCOTTINI et al., 2015). The sequence of collection by cytology is described in Figure 2.

Figure 2. Scheme of performing the cytology technique for dairy cows.

(1) Cytological brush attached to the artificial insemination pipette protected with a plastic sheath protector, covered by a sanitary sheath during insertion in the vagina; (2) After passing through the cervix, the cytobrush is exposed in the uterine body, then the cytobrush is rolled three times against the wall of the uterine body; (3) Then, the cytobrush is exteriorized with the collected material; (4) After that, the cytobrush is spread over the microscopic slide; (5) Then, the slide is air-dried and stained with cytology-specific staining; (6) Finally, the proportion of the PMNL cells is counted in an optical microscope.

Fonte: Paiano, R. B. (2021).

Early diagnosis of uterine diseases is necessary to reduce the negative impact caused by these diseases on the profitability of farms (ABUELO et al., 2021). Previous studies have shown that cows that develop uterine diseases during the puerperal phase may show some signs such as reduced dry matter intake (HUZZEY et al., 2007) or hematological (HAMMON et al., 2006) and biochemical (PAIANO et al., 2021) alterations, before presenting clinical signs of diseases such as vaginal discharge, this monitoring facilitates the identification of these animals.

HUZZEY et al., (2007) observed that for each 10-minute reduction in the average daily feeding time during the last week before parturition, the odds of the cow developing postpartum uterine diseases increased by 1.72 times, in addition, the authors identified that for each 1 kg reduction in dry matter intake during this period cows were almost 3 times more likely to be diagnosed with uterine diseases.

In another recent study PAIANO et al., (2021) identified that cows that develop uterine diseases during the puerperium show biochemical alterations during the three weeks prior to parturition. The authors reported a lower concentration of urea and creatinine for cows that developed uterine diseases that may be related to lower dry matter intake; increase in the serum concentration of fibrinogen, which is considered a positive marker of inflammation, showing an exacerbated pro-inflammatory status in these animals; in addition, a higher serum concentration of β-hydroxybutyrate and non-esterified fatty acids was noted, which is suggestive of an exacerbated negative energy balance in these animals (PAIANO et al., 2021).

In this sense, the monitoring and evaluation of dairy cows during the puerperium phase, in addition to carrying out the diagnosis correctly, is essential for making early decisions on farms, to reduce the economic losses caused by uterine diseases.

2.2 Therapy of uterine diseases

The use of antibiotics is the most used treatment in uterine diseases, with cephapirin and ceftiofur being the main drugs selected in the therapy of these diseases (LEBLANC et al., 2002; DUBUC and DENIS-ROBICHAUD, 2017; MORAES et al., 2017). However, the indiscriminate use of antibiotics in dairy farms favors the development of pathogenic bacteria resistance to drugs (SANTOS et al., 2011), impairing the success of drugs, contributing to the failure of therapeutic success (LIU et al., 2009). In this sense, studies involving new alternatives as a form of non-conventional therapy (without the use of antibiotics) for uterine diseases in dairy cows have been increasing in recent years (ZINICOLA et al., 2018; GALVÃO et al., 2019; MEIRA JUNIOR et a., 2020).

Among the forms of non-conventional therapies, those obtained from natural sources have a promising prominence mainly for not causing the disposal of milk and also not favoring the resistance of bacteria. In this sense, the essential oils that are volatile compounds naturally produced by plants appear as a promising source for treating diseases due to their excellent antimicrobial activity (BONILLA et al., 2018). Essential oils can be extracted from different parts of plants such as flowers, leaves, fruits, seeds, roots, stems and barks (BAKKALI et al., 2008; SZWEDA et al., 2018). Essential oils are widely used in the food, beverage, cosmetics and pharmaceutical industries, in addition they are classified as GRAS (generally regarded as safe), showing excellent antibacterial, antifungal, antiviral, antiparasitic, insecticide, antiprotozoal activities, among others (BONILLA and SOBRAL, 2019).

Recently, some studies have demonstrated the antimicrobial activity of natural products using essential oils against bovine diseases including clinical (DAL POZZO et a., 2011) and subclinical (CHO et al., 2011) mastitis, diarrhea (KATSOULOS et a., 2017) and pneumonia (AMAT et al., 2019). However, the literature lacks information on the antimicrobial activity of essential oils against pathogens in cows with uterine diseases. PAIANO et al., (2020d) recently evaluated the *in vitro* activity of natural therapy using seven essential oils against the main pathogens that cause uterine diseases in dairy cows. In this study, the antimicrobial activity of the essential oils of rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum cassia*), clove (*Eugenia caryophyllus*), eucalyptus (*Eucalyptus globulus*), lemon (*Citrus limon*), oregano (*Origanum vulgare*) and white thyme (*Thymus vulgaris*) were tested by the disk diffusion assay against the reference strain of *Trueperella pyogenes* (ATCC 19411), *Escherichia coli* (ATCC

25922) and *Fusobacterium necrophorum* (ATCC 25286) (PAIANO et al., 2020d), the results and characteristics of the essential oils are shown in Table 2.

Table 2. Chemical composition of various essential oils and their antibacterial activity against uterine diseases pathogens.

Essential oil	**Part used**	**Major chemical compounds**	**Inhibited microorganisms**
Cinnamomum cassia	Leaves and bark	Cinnamaldehyde, coumarin, styrene and bezaldehyde	*T. pyogenes*, *E. coli* and *F. necrophorum*
Citrus limon	Fruits	Limonene, β-pinene and γ-terpinene	-
Eucalyptus globulus	Leaves	1,8-cineol, limonene, α-pinene and γ-terpinene	*E. coli*
Eugenia caryophyllus	Leaves	Eugenol and β-caryophyllene	*T. pyogenes*, *E. coli* and *F. necrophorum*
Origanum vulgare	Flowers	Carvacrol, *p*-cymene, β-caryophyllene, linalool, γ-terpinene and thymol	*T. pyogenes*, *E. coli* and *F. necrophorum*
Rosmarinus officinalis	Leaves	1,8-cineol, α-pinene, β-pinene, camphor and camphene	*E. coli*
Thymus vulgaris	Leaves and flowers	Thymol, *p*-cymene, γ-terpinene and linalool	*T. pyogenes*, *E. coli* and *F. necrophorum*

One of the mechanisms of action of essential oils includes cell wall degradation, denaturation and coagulation protein, thus altering the permeability of the cytoplasmic membrane contributing to cell death (BENCHAAR et al., 2008). Another reported mechanism is based on the modification of ion gradients, which can contribute to the deterioration of electron transport, translocation of proteins, causing the loss of chemiosmotic control of the affected cell, leading to the death of the bacteria (DORMAN and DEANS, 2000). The last mechanism described occurs due to the action caused by damage to the cytoplasmic membrane, which can interfere with the integrity and functioning of the cell membrane, changing the potential of the membrane, contributing to the loss of cytoplasmic material and inhibition of the respiratory chain, damaging the membrane proteins, which can result in the extravasation of cellular content, cytoplasmic coagulation and exhaustion of the proton pumping system (TAKZAREE et al., 2017).

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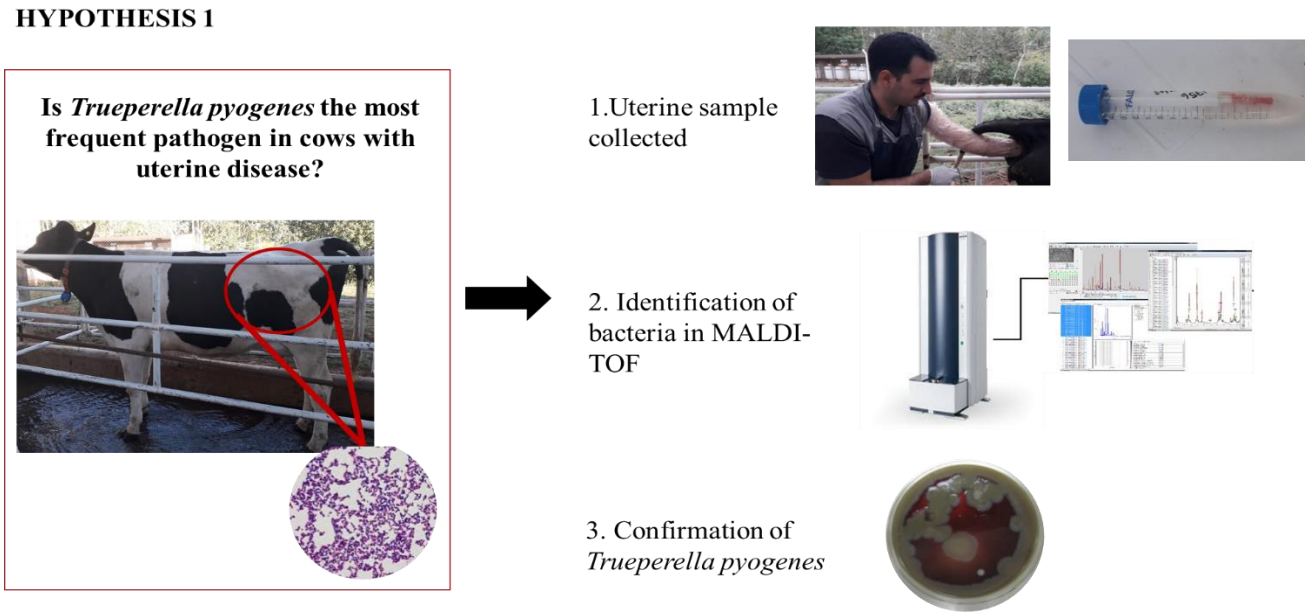
3 HYPOTHESES

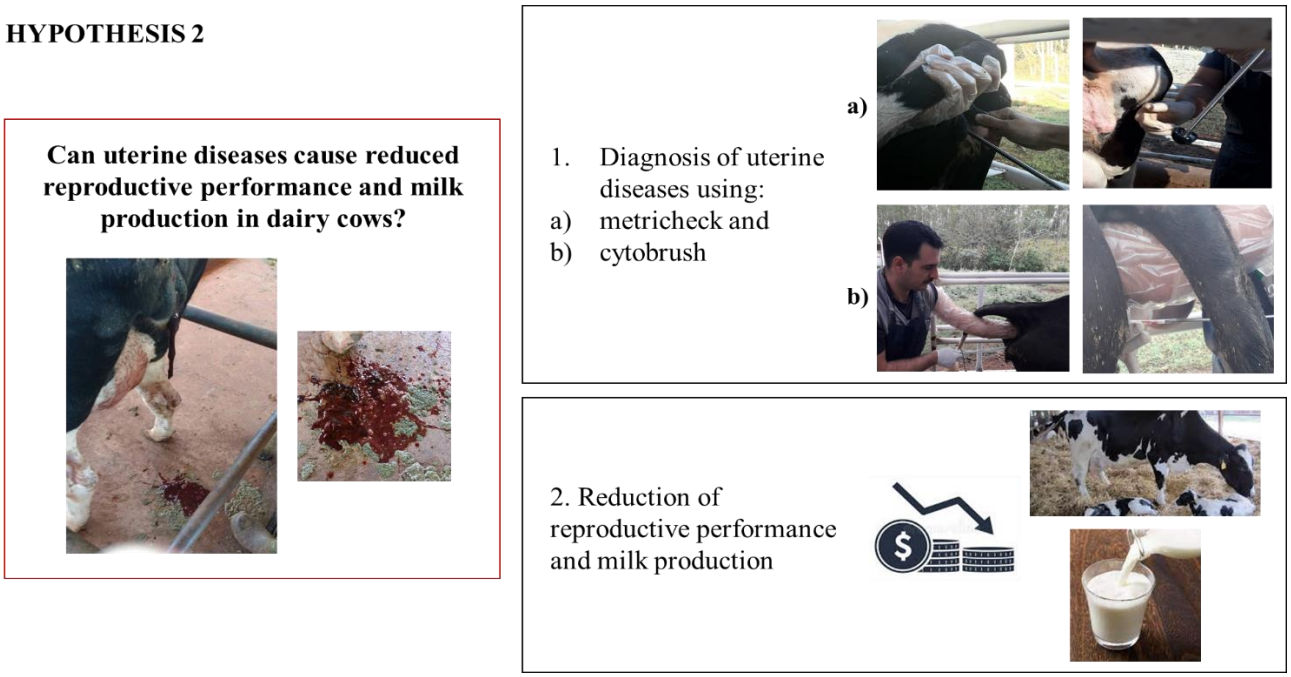
Cows with uterine diseases present higher frequency of the pathogen *Trueperella pyogenes* in the uterine environment (Figure 3). Uterine diseases cause reduced reproductive performance and milk production in dairy cows in tropical conditions (Figure 4). Essential oils have *in vitro* antimicrobial activity against standard bacterial strains of the main bacteria causing endometritis (*Escherichia coli* ATCC 25922, *Fusobacterium necrophorum* ATCC 25286 and *Trueperella pyogenes* ATCC 19411) (Figure 5).

Figure 3. Hypothetical model design of study 1.



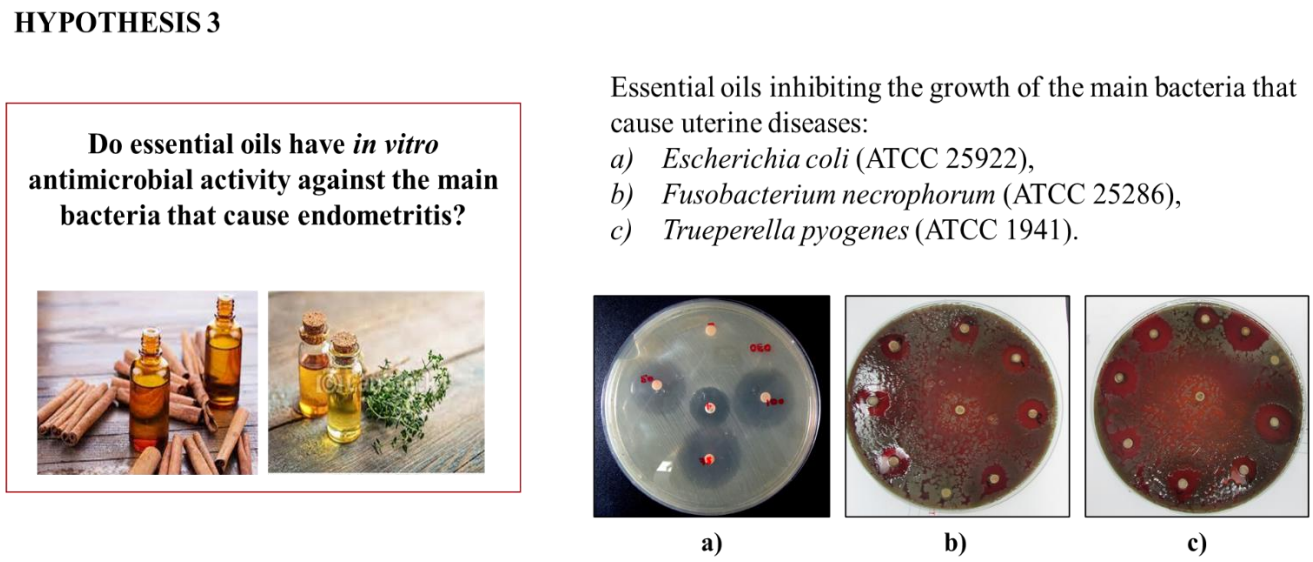
Fonte: Paiano, R. B. (2021).

Figure 4. Hypothetical model design of study 2.



Fonte: Paiano, R. B. (2021).

Figure 5. Hypothetical model design of study 3.



Fonte: Paiano, R. B. (2021).

4 OBJECTIVES

3.1 Identify the main microorganisms present in the uterine environment of dairy cows with uterine diseases during the puerperium period.

3.2 Identify the main factors associated with the occurrence of uterine diseases in dairy cows and evaluate the impact of reproductive performance and milk production of cows affected by uterine diseases.

3.3 To evaluate the *in vitro* antibacterial action by disk diffusion assay of seven essential oils (rosemary, cinnamon, cloves, eucalyptus, lemon, oregano and thyme) against the standard strains of *Escherichia coli (*ATCC 25922), *Fusobacterium necrophorum* (ATCC 25286) and *Trueperella pyogenes* (ATCC 19411).

STUDY 1: COMPARISON OF THE MAIN MICROORGANISMS ASSOCIATED WITH CLINICAL AND SUBCLINICAL ENDOMETRITIS BY MATRIX-ASSISTED LASER DESORPTION IONIZATION-TIME OFF LIGHT MASS SPECTROMETRY

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5 STUDY 1: COMPARISON OF THE MAIN MICROORGANISMS ASSOCIATED WITH CLINICAL AND SUBCLINICAL ENDOMETRITIS BY MATRIX-ASSISTED LASER DESORPTION IONIZATION-TIME OFF LIGHT MASS SPECTROMETRY

5.1 ABSTRACT

Clinical endometritis (CE) and subclinical endometritis (SE) are diseases that affect dairy cows during the puerperium causing negative effects on the animals' milk production and fertility. The objective of this study was to assess the main microorganisms related to cases of CE and SE from uterine samples of dairy cows in Brazilian herds. Selective and differential media were used for isolation of aerobic and anaerobic bacteria and further Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) identification. A total of 279 lactating dairy cows with 28 to 33 days in milk from six commercial farms were evaluated. Initially, cows were classified in three groups: cytologic healthy cows (n = 161), cows with CE (n = 83), and cows with SE (n = 35). A total of 127 bacterial species of 48 genera were identified. Healthy animals presented 97 species, followed by the CE group with 53 identified species, while SE cows presented only 21 bacterial species. There was a significantly higher isolation rate of *Trueperella pyogenes* in CE (26.5%) cows compared to healthy and SE cows. Some anaerobic species were exclusively isolated from the CE group, even though presented lower frequency. Interestingly, 18.1% of samples from CE cows and 40% of SE cows were negative to bacterial isolation. These findings could represent the basis for the development of future treatment strategies.

Keywords: clinical endometritis, subclinical endometritis, uterine microbiota, dairy cow, MALDI-TOF MS

5.2 INTRODUCTION

Endometritis is one of the most important causes of infertility in dairy herds. It contributes to the reduction of profitability in dairy farms (PÉREZ-BÁEZ et al., 2021), mainly due to the costs related to the therapy of the affected animals, milk disposal, contributing to the decrease in milk production (PAIANO et al., 2019) and worsened fertility of the affected cows (GILBERT et al., 2005; LEBLANC, 2008; PAIANO et al., 2020). Endometritis is the inflammation of the endometrium, the innermost lining of the uterus, being subdivided into clinical endometritis (CE) and subclinical endometritis (SE) (SHELDON et al., 2006). Clinical endometritis is defined as the presence of purulent or mucopurulent vaginal discharge detected three weeks or more postpartum (LEBLANC et al., 2002). Subclinical endometritis is characterized by an increased proportion of polymorphonuclear neutrophil leukocytes (PMNL) in the endometrium in the absence of clinical disease (GILBERT et al., 2005).

Previous studies have reported that the main bacterial species associated with endometritis in dairy cows are *Trueperella pyogenes*, *Escherichia coli*, and *Fusobacterium necrophorum* (WILLIAMS et al., 2005; SHELDON et al., 2010; MACHADO et al., 2012; BICALHO et al., 2017a; PASCOTTINI et al., 2021). In addition, the negative impact of the presence of *Trueperella pyogenes* in the uterine environment of dairy cows affected by endometritis on reproductive performance and milk production was demonstrated in a recent study carried out in Brazil (PAIANO et al., 2021).

Currently, the main techniques described for the identification of uterine microorganisms involve culture-dependent methods or metagenomic analysis. When the studies involve culture and identification of isolated bacteria, it can be conducted using traditional biochemical methods or more modern techniques like matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (BALLAS et al., 2021), sequencing of 16S rRNA gene (PASCOTTINI et al., 2020), or Fourier transform infrared (FTIR) (PRUNNER et al., 2014; WAGENER et al., 2014). Metagenomic analyses are conducted via 16S rRNA gene profiling by high-throughput sequencing (BICALHO et al., 2017b; WANG et al., 2018).

Although molecular technique has succeeded in diagnosing of uterine pathogens, the challenges of achieving proper molecular identification and serotyping at the lowest possible cost still remain. In recent years, MALDI-TOF MS has become an important bioanalytical diagnostic tool for the detection of protein profiles from whole bacterial cells

(CARBONNELLE et al., 2011). This rapid and accurate method can be easily applied to identify bacteria at the genus, species and, in some cases, the subspecies levels (KLIEM et al., 2012). Therefore, MALDI-TOF MS represents a promising alternative to the standard phenotypic and molecular techniques carried out in diagnostic laboratories. In the present study, we assessed the use of MALDI-TOF MS to identify the main microorganisms related to cases of CE and SE from uterine samples of dairy cows. The clear identification of microorganisms related to endometritis in dairy cows may allow for a lower amount of antibiotics used in dairy herds, contributing to specific strategic therapies for the main microorganisms on each farm.

5.3 MATHERIALS AND METHODS

5.3.1 Study farms

The study was conducted between May and November 2019 on six commercial dairy farms in São Paulo State, Brazil. Holstein lactating dairy cows were housed in freestall barns. Cows were fed twice daily with a diet of total mixed ration (TMR) consisting of corn silage, with cornmeal, soybean meal, and mineral supplements, formulated according to NRC (2001) to meet the nutritional requirements for lactating Holstein cows. Cows were milked twice daily. This study was approved by the ethics committee for the use of animals of FMVZ-USP, approval No. 1377270218.

5.3.2 Sampling and case classification

A total of 279 cows were examined. It was a convenience sample of which the evaluated animals were originally sampled for the assessment of healthy and diseased individuals based on previously reported endometritis incidence of 23.8% and 95% confidence level with 5% precision (THRUSFIELD, 2005). Only cows that were not treated with antibiotics drugs or non-steroidal or steroidal anti-inflammatory drugs after parturition were included in this study. In addition, the cows sampled in the present study showed no clinical signs of clinical mastitis, milk fever, ketosis.

All lactating cows included in this study were evaluated at 28 and 33 days after calving. Initially, the perineal area of the cow was cleansed with 70% ethylic alcohol and dried using a paper towel, then the vaginal discharge score (VDS) was evaluated using the metricheck device

(Simcro Tech, Hamilton, New Zealand) and scored as 0 = clear mucus, 1 = mucus with flecks of pus, 2 = mucopurulent discharge (≤ 50% pus), and 3 = purulent discharge (> 50% pus). Next, cytology and bacteriological samples of the uterus were collected, according to the technique described by KASIMANICKAM et al. (2004). A sterile cytobrush rod (covered with a sterile sanitary sheath) was introduced into the vagina and guided through the cervix per rectum, as described by PASCOTTINI et al. (2020). Once outside the genital tract, the cytobrush was gently rolled onto a sterilized microscope slide. The first 5 cm of the cytobrush was then cut with sterile scissors, placed in a sterile 15 mL plastic conical tube with anaerobic transport medium, and kept at 4 °C until arrival at the laboratory for bacteriological examination. Cytology slides were stained using the panoptic rapid staining method (Laborclin ®, Pinhais, Brazil). Evaluations were performed under a microscope at 400× magnification (Nikon, E200, Tokyo, Japan). A total of 200 cells were counted in the cytological slide to determine the proportion of PMNL (PASCOTTINI et al., 2020).

Animals were classified in three groups based on uterine health considering vaginal discharge and cytological analysis: healthy cows (without CE and without SE, n = 161), cows with CE (vaginal discharge score = 3 and > 18% PMNL, n = 83) and cows with SE (vaginal discharge 0, 1 or 2, and > 18% PMNL, n = 35) (KASIMANICKAM et al., 2004). Four cows with VDS score = 3 and PMNL < 18 that were considered to have cervicitis and were not included in the study.

5.3.3 Bacterial isolation

Immediately after sampling, the cytobrushes were transferred to 6 mL of anaerobic transport medium. At the laboratory, the cytobrushes were washed in 2 mL of *Brucella* broth (Difco-BBL, Sparks, MD, USA). Then, 10 µL of broth was streaked onto *Brucella* agar (Difco-BBL, Sparks, MD, USA) with 7% whole defibrinated horse blood, supplemented with hemin (5 mg l−1) and menadione (1 mg l−1), and plates were incubated a 37 °C for 48 h under anaerobic conditions. An aliquot of 10 µL of broth was also streaked onto MacConkey agar, CHROMagar^{TM} Orientation and blood agar with 5% defibrinated sheep blood (Difco-BBL, Sparks, MD, USA). These plates were incubated at 37 °C for 24-48 h under aerobic conditions. All morphologically different colonies were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and stored at −86°C in brain-

heart infusion medium (Difco, Sparks, MD, USA) with 30% of glycerol, supplemented with fetal calf serum (5%).

Identification of the bacteria by MALDI-TOF MS was carried out as described previously by HIJAZIN et al. (2012). Mass spectra were acquired using a MicroflexTM mass spectrometer (Bruker Daltonik) and identified with the manufacturer's software MALDI BioTyperTM 3.0. The requirements for interpreting the standards of the manufacturer Bruker Daltonik were used in this study as follows: scores ≥ 2.0 were accepted for species assignment and scores ≥ 1.7 and ≤ 2.0 were used for genus identification.

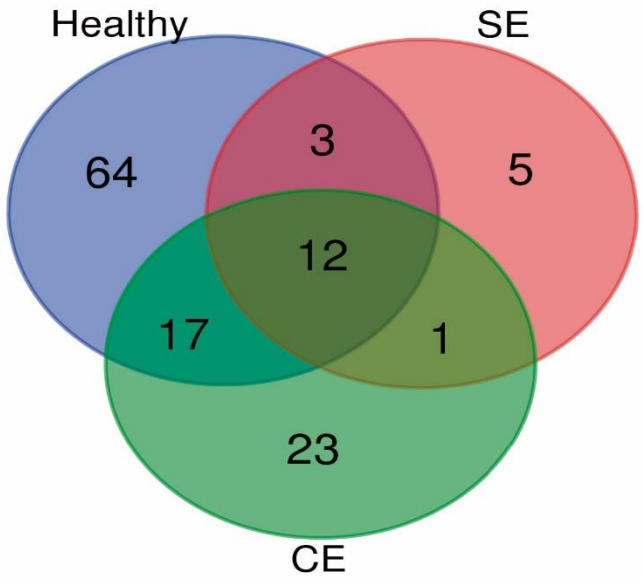
5.3.4 Statistical analysis

A Chi-square test was deployed to compare the percentage frequencies of bacteriological findings in healthy, CE, and SE groups. In cases where the expected value was ≤ 5, the Fisher's Exact Test was used. The Venn diagram was constructed using an online tool http://bioinformatics.psb.ugent.be/webtools/Venn/ (Bioinformatics and Evolutionary Genomics Group, Ghent University).

5.4 RESULTS

The frequency of samples without bacterial isolation was 40% (14/35) in SE and 35.4% (57/161) in healthy animals, greater than 18.1% (15/83) found in the CE group (P = 0.009). A total of 127 bacterial species of 48 genera were identified in these three groups of animals. Healthy animals presented numerically greater richness (97 identified species) followed by the CE group (53 identified species), while only 21 bacterial species were identified among the SE group. However, most of the species from the SE group are shared between healthy animals and those with CE (Figure 6).

Figure 6. Venn diagram illustrating the common and exclusive bacterial species isolated from the uterine samples collected of the three groups (healthy, CE, and SE).



Healthy
Number 64
Acinetobacter schindleri
Actinomyces odontolyticus
Atopobium minutum
Bacillus altitudinis
Bacillus clausii
Bacillus coagulans
Bacillus oleronius
Bacillus pumilus
Bacillus thuringiensis
Brachybacterium conglomeratum
Brevibacillus agri
Brevibacillus laterosporus
Brevibacillus parabrevis
Brevibacterium luteolum
Cellulosimicrobium cellulans
Citrobacter amalonaticus
Citrobacter freundii
Citrobacter koseri
Corynebacterium flavencens
Corynebacterium glutamicum
Corynebacterium jeikeium
Corynebacterium stationis
Cutibacterium acnes
Enterobacter kobei
Enterobacter xiangfangensis
Enterococcus avium
Enterococcus casseliflavus
Enterococcus durans
Enterococcus faecalis
Enterococcus italicus
Enterococcus mundtii
Enterococcus saccarolyticus
Enterococcus thailandicus
Escherichia hermannii
Klebsiella variicola
Kocuria marina
Kocuria rhizophila
Leclercia adecarboxylata
Lysinibacillus fusiforms
Macrococcus canis
Microbacterium esteraromaticum
Microbacterium oxydans
Microbacterium paraoxydans
Micrococcus lylae
Paenibacillus amynolyticus
Paenibacillus barcinonensis
Paenibacillus illinoisensis
Pantoea agglomerans
Pantoea ananatis
Pluralibacter gergoviae
Proteus hauseri
Pseudomonas putida
Roseomonas mucosa
Salmonella enterica
Staphylococcus caprae
Staphylococcus sciuri
Staphylococcus cohnii
Staphylococcushaemolyticus
Streptococcus alactolyticus
Streptococcus dysgalactiae
Streptococcus lutetiensis
Streptococcus lutetiensis
Streptococcus mitis
Vagococcus fluvialis

CE
Number 23
Acinetobacter beijerinakii
Actinomyces hyovaginalis
Bacteroides pyogenes
Clostridium diolis
Dermabacter hominis
Enterococcus aerogenes
Enterococcus villorum
Fusobacterium necrophorum
Helcococcus kunzii
Helcococcus ovis
Histophilus somni
Klebsiella pneumoniae
Lactobacillus agilis
Lysinibacillus xylanilyticus
Paenibacillus residui
Paenibacillus turicensis
Porphyromonas levii
Prevotella heparinolytica
Pseudomonas aeruginosa
Rothia amarae
Streptococcus pneumoniae
Streptococcus salivarius
Streptococcus sanguinis

Healthy x SE
Number 3
Corynebacterium efficiens
Paenibacillus phoenicis
Staphylococcus chromogenes

CE x SE
Number 1
Pseudomonas rhodesiae

SE
Number 5
Acinetobacter radioresistens
Bacillus megaterium
Bacillus siralis
Scedosporium apiospermum
Streptococcus uberis

CE x Healthy
Number 17
Bifidobacterium pseudolongum
Corynebacterium xerosis
Delftia acidovorans
Enterobacter cloacae
Enterococcus faecium
Enterococcus gallinarum
Enterococcus hirae
Klebsiella oxytoca
Lysinibacillus massiliensis
Paenibacillus macerans
Pseudomonas monteilii
Siccibacter turicensis
Staphylococcus arlettae
Staphylococcus epidermidis
Staphylococcus hominis
Staphylococcus xylosus
Trueperella pyogenes

CE x Healthy x SE
Number 12
Aerococcus viridans
Bacillus cereus
Bacillus licheniformis
Clostridium perfringens
Escherichia coli
Micrococcus luteus
Paenibacillus cookii
Pseudomonas fulva
Pseudomonas stutzeri
Staphylococcus warneri
Stenotrophomonas maltophilia
Streptococcus pluranimalium

Also, the bacterial species common to all three groups include the most prevalent microorganisms among studied animals, such as *Escherichia coli*, *Aerococcus viridans*, and *Bacillus cereus* (Table 3). Interestingly, *Trueperella pyogenes* was the most frequent microorganisms among the CE group (32.4%, 22/68) differing ($P < 0.001$) from the SE and healthy groups, respectively (Table 3).

Table 3. Bacterial population isolated from healthy dairy cows and dairy cows with clinical (CE) and subclinical (SE) endometritis – N (%).

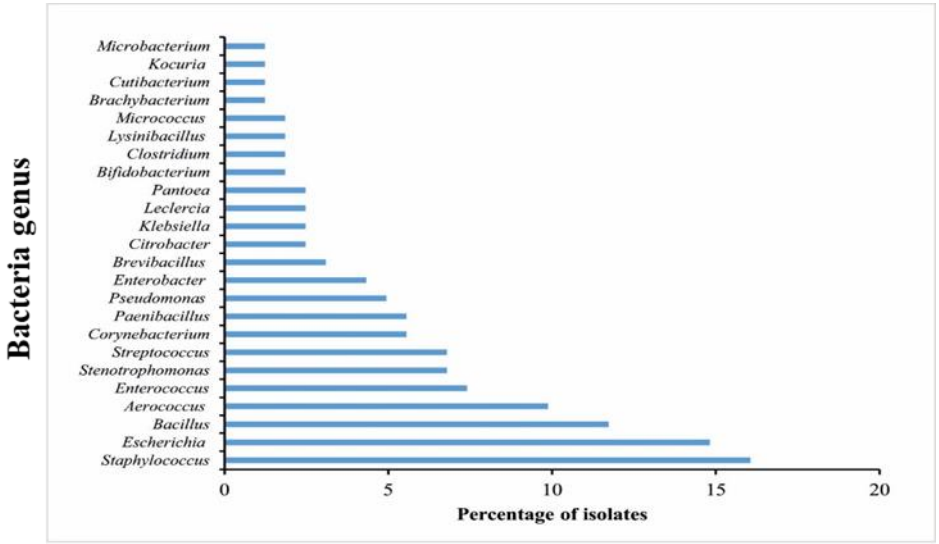
Bacteria	CE (N = 83)	SE (N = 35)	Healthy (N = 161)
Trueperella pyogenes	22 (32.4)[a]	0 (0.0)[b]	1 (0.6)[b]
Escherichia coli	16 (19.3)	2 (5.7)	24 (14.9)
Bacillus spp.	14 (16.9)	8 (20.0)	21 (13.0)
CNS	14 (16.9)	2 (5.7)	32 (19.9)
Aerococcus viridans	11 (13.3)	5 (14.2)	16 (9.9)
Pseudomonas spp.	7 (8.4)	4 (10.0)	8 (5.0)
Enterococcus spp.	6 (7.2)	0 (0.0)	19 (11.8)
Helcococcus spp.	5 (6.0)	0 (0.0)	0 (0.0)
α-Hemolytic streptococci	5 (6.0)	3 (7.5)	9 (5.6)
Paenibacillus spp.	4 (4.8)	3 (7.5)	11 (6.8)
Prevotella heparinolytica	4 (4.8)	0 (0.0)	0 (0.0)
Bacteroides pyogenes	3 (3.6)	0 (0.0)	0 (0.0)
Klebsiella spp.	3 (3.6)	0 (0.0)	4 (2.5)
Lysinibacillus spp.	3 (3.6)	0 (0.0)	3 (1.9)
Porphyromonas levii	3 (3.6)	0 (0.0)	0 (0.0)
Bifidobacterium pseudolongom	2 (2.4)	0 (0.0)	3 (1.9)
Candida spp.	2 (2.4)	0 (0.0)	0 (0.0)
Clostridium spp.	2 (2.4)	1 (2.8)	3 (1.9)
Corynebacterium spp.	2 (2.4)	1 (2.8)	10 (6.2)
Delftia spp.	2 (2.4)	0 (0.0)	1 (0.6)
Enterobacter spp.	2 (2.4)	0 (0.0)	7 (4.3)
Fusobacterium necrophorum	2 (2.4)	0 (0.0)	0 (0.0)
Micrococcus luteus	2 (2.4)	1 (2.8)	3 (1.9)
Stenotrophomonas maltophilia	2 (2.4)	1 (2.8)	11 (6.8)
Acinetobacter spp.	1 (1.2)	1 (2.8)	1 (0.6)
Actinomyces spp	1 (1.2)	0 (0.0)	1 (0.6)
Dermabacter hominis	1 (1.2)	0 (0.0)	0 (0.0)
Histophilus somni	1 (1.2)	0 (0.0)	0 (0.0)
Lactobacillus agilis	1 (1.2)	0 (0.0)	0 (0.0)
Rothia amarae	1 (1.2)	0 (0.0)	0 (0.0)

Siccibacter turicensis	1 (1.2)	0 (0.0)	1 (0.6)
No isolation	15 (18.1)[b]	14 (40.0)[a]	57 (35.4)[a]

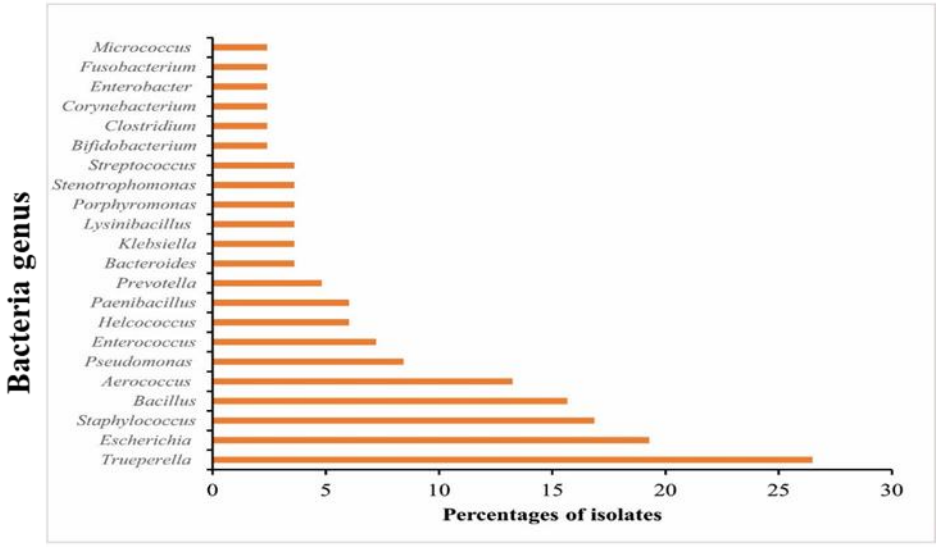
[a,b] Values within a row with different superscript letters differ at $P < 0.05$. CNS - coagulase-negative *Staphylococcus*.

Considering the healthy animals, isolates from the *Staphylococcus* genus were most frequently detected (18.6%; 30/161), followed by isolates belonging to the genera *Escherichia* (14.9%; 24/161), *Bacillus* (12.4%; 20/161), *Enterococcus* (11.1%; 18/161), and *Aerococcus* (10.6%; 17/161). For CE cows, the *Trueperella* genus was the most frequent (26.5%; 22/83), followed by isolates belonging to the genera *Escherichia* (19.3%; 16/83), *Staphylococcus* (16.9%; 14/83), *Bacillus* (14.4%; 12/83), *Aerococcus* (13.3%; 11/83), and *Pseudomonas* (8.4%; 7/83), respectively. Finally, concerning cows with SE, isolates of the *Bacillus* genus were detected more frequently (22.9%; 8/35), followed by the genera *Aerococcus* (14.3%; 5/35), *Pseudomonas* (11.4%; 4/35), *Streptococcus* (8.6%; 3/35), and *Paenibacillus* (8.6%; 3/35), respectively (Figure 7).

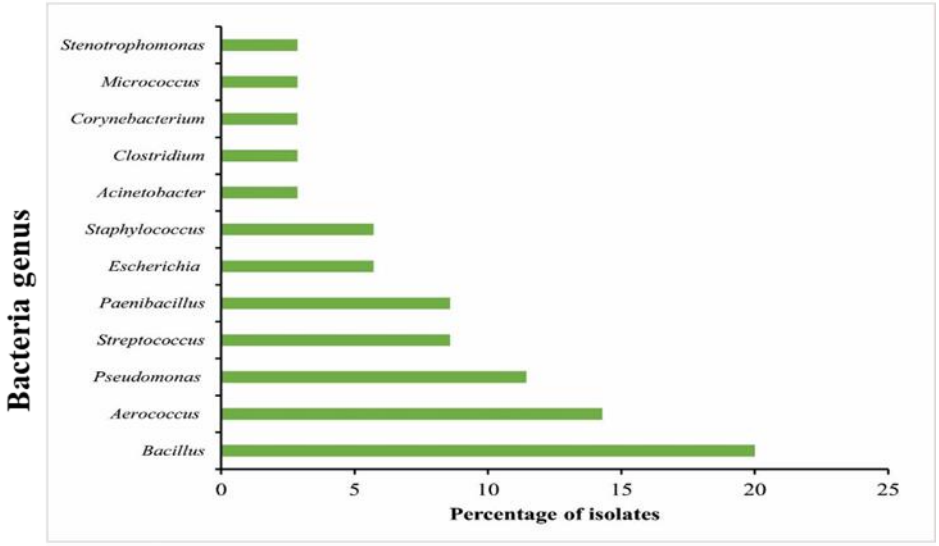
Figure 7. Frequency of different genera isolated from (A) healthy animals, (B) CE cows, and (C) SE cows.



A



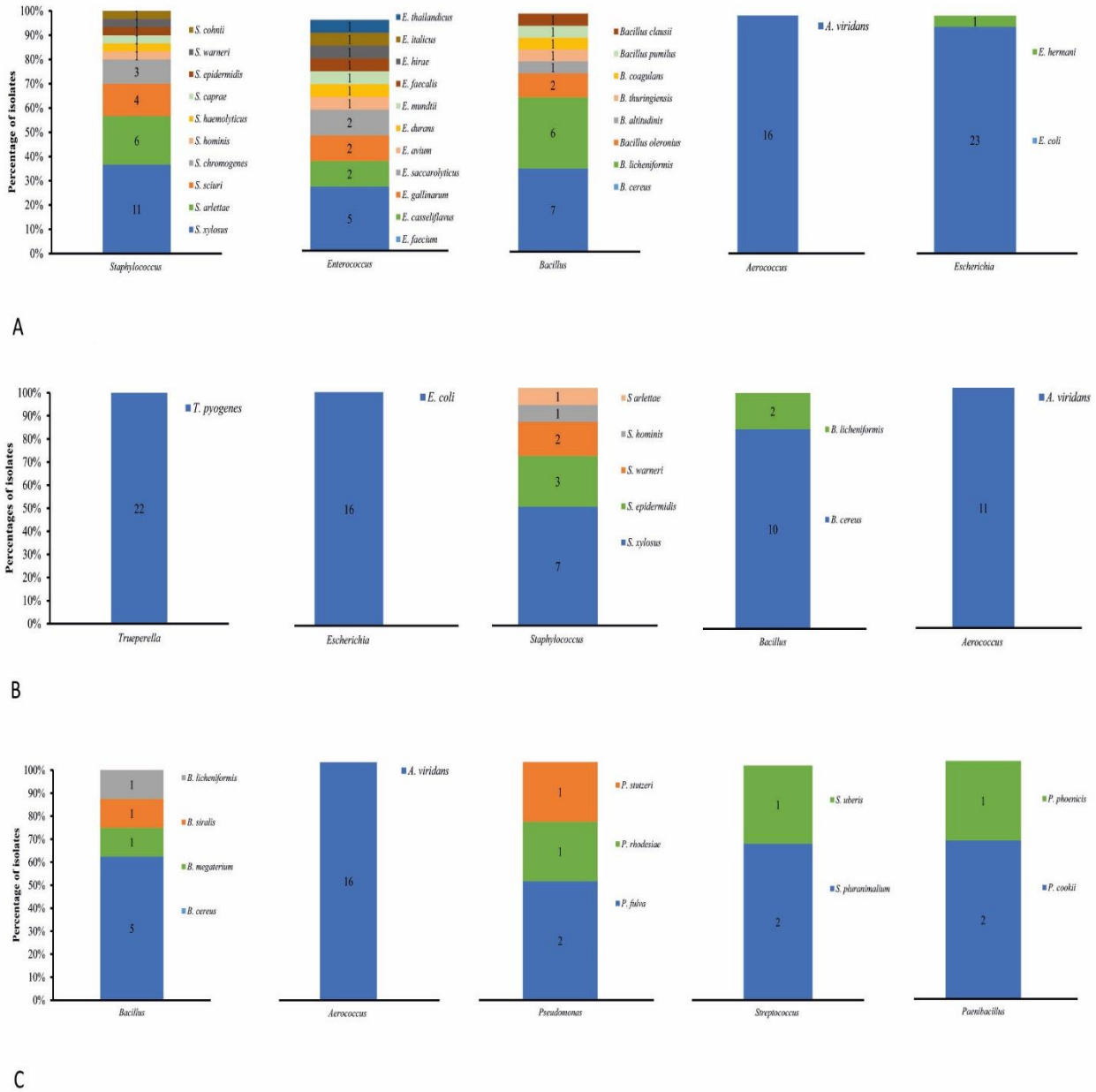
B



C

The most frequent species belonging to the predominant genera for healthy, CE and SE cows are represented in Figure 8.

Figure 8. Most frequently isolated genera that were shown on the species level. Stacked bars represent the relative frequency of microorganisms isolated from (A) healthy cows, (B) CE cows, and (C) SE cows. The numbers within the stacked bars indicate the absolute number of isolates of each species.

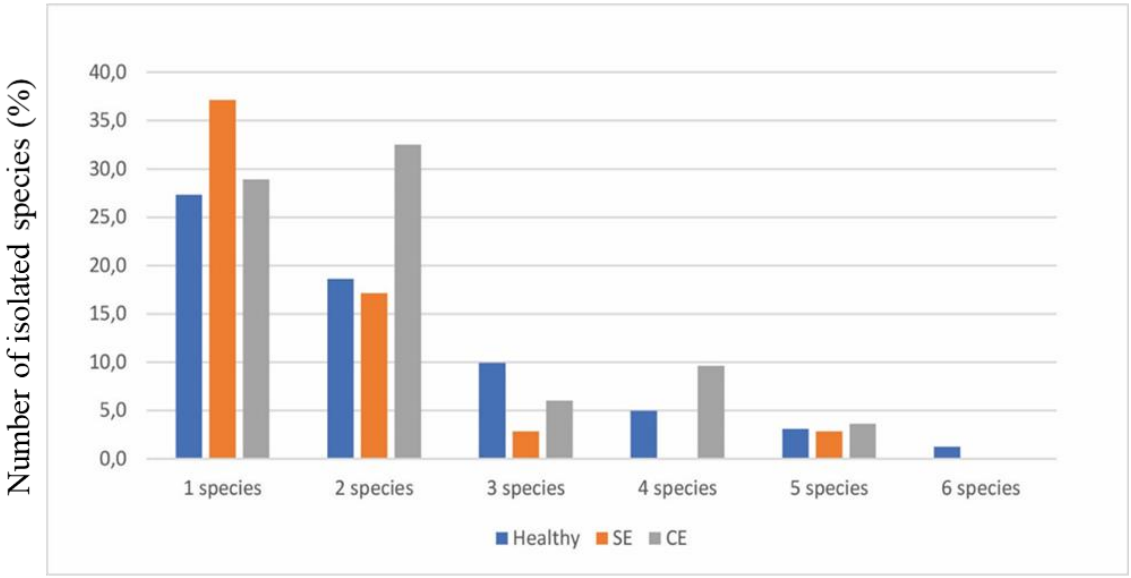


It is highlighted the greater richness of the *Enterococcus*, *Staphylococcus,* and *Bacillus* genera. For the healthy cows, the *Enterococcus* genus represented the most varied group, including 11 different species, with *E. faecium* (5/18; 27.8%) being the most prevalent. Next, the *Staphylococcus* and *Bacillus* genera were composed of 10 and 8 different species, respectively, with a predominance of *S. xylosus* (11/30; 36.7%) and *B. cereus* (7/20; 35.0%). Regarding cows with CE, the *Staphylococcus* genus represented the most heterogeneous group including five different species. Meanwhile, in the SE group, the *Bacillus* genus was the most diverse, including four different species with a predominance of *B. cereus* (5/8; 62.5%). Interestingly, for the *Aerococcus* genus, only *A. viridans* was identified and presented a high frequency among all studied groups.

There was a significant higher isolation rate (P = 0.00024) of *T. pyogenes* in the CE group (26.5%; 22/83) compared to healthy (0.6%; 1/161), and SE cows (0.0%; 0/35). For other genera or species, no significant differences were found in the percentages between healthy, CE, and SE groups. Other species as *Prevotella heparinolytica*, *Porphyromonas levii*, *Fusobacterium necrophorum*, *Bacteroides pyogenes*, and *Helcococcus* spp. were exclusively isolated at the CE group, even though they presented lower frequency. Interestingly, these bacteria were isolated in association with *T. pyogenes*. Of the total samples with *T. pyogenes* isolation (n = 22), in four samples we observed this agent as a monoculture, six samples presented an association with *E. coli,* and in 12 samples the agent was associated with different bacterial species (Table S1).

The number of bacterial species isolated according to the health status of animals is presented in Figure 9 and the respective isolation profiles are described in Tables S2 and S3. There was a predominance of monocultures for the healthy and SE groups (42.3% and 61.9%, respectively), while the cows with CE presented a high frequency of infections caused by two distinct bacterial species (39.7%) and monocultures (36.8%), respectively. Among the detected monocultures, *A. viridans* and *B. cereus* predominated among the three groups, and *E. coli* and *S. xylosus* were shared between the healthy and CE groups; while *T. pyogenes* and *S. maltophilia* and *S. pluranimalium* were exclusively detected in the CE and healthy cows, respectively (Tables S1 and S2). High heterogeneity of isolation profiles was observed for the healthy cows.

Figure 9. Number of different bacterial species isolated in each sample from healthy, CE, and SE cows.



5.5 DISCUSSION

There is a growing interest in the microbiota of the reproductive tract of dairy cows. Studies in this field are fundamental for the knowledge of the most frequent microorganisms that can cause uterine diseases and affect the fertility of dairy cows. Also, more studies will contribute to new therapies focused exclusively on the main microorganisms associated with uterine diseases.

Previously, the uterus was believed to be sterile during pregnancy and contaminated with non-specific bacteria after parturition. However, there is now evidence from different studies that the uterus is not sterile (SHELDON et al., 2019). Specific microorganisms are adapted to the endometrium and can also influence the uterine cells present in the micro-environment. Modern culture-independent molecular methods focused on sequencing have also widened our present understanding of the microbiome of the uterus in cattle with metritis, pyometra, and endometritis (APPIAH et al., 2020).

Our findings reveal a diverse community of 127 different species representing 48 genera. Among them, the most prevalent genera were *Escherichia*, *Staphylococcus*, *Bacillus,* and *Aerococcus* and the most prevalent species were *Escherichia coli* and *Aerococcus viridans.* The results of the present study are corroborated by WAGENER et al. 2015, who reported a diverse uterine microbiota consisting of 202 different species and 76 genera, and Ballas et al. (2021) who identified 116 different species and 49 genera. BALLAS et al. (2021) identified a high prevalence of bacteria of *Bacillus* and *Staphylococcus* genera in cows with and without endometritis at the time of AI in 120 dairy cows from Slovakia. However, the same authors identified a low prevalence of *Trueperella* and *Escherichia* in uterine isolates. In Austria, WAGENER et al. (2015) reported *Staphylococcus*, *Trueperella,* and *Escherichia* as the most prevalent genera in cows with and without endometritis during the puerperium period in 122 cows evaluated.

Our study showed that the most frequent bacteria isolated from CE cows were *T. pyogenes*. These findings agree with previous studies conducted in Europe (WERNER et al., 2012; WAGENER et al., 2014). Using culture-independent methods, PASCOTTINI et al. (2020) observed that cows with CE had microbiota characterized by a greater relative abundance of *Fusobacterium* and *Trueperella* and a lower relative abundance of *Escherichia, Shigella, Lactobacillus, Prevotella, Schlegelella, Staphylococcus,* and *Streptococcus* than healthy and SE cows. Furthermore, as observed by other authors (WANG et al., 2018), no difference was noted between the groups evaluated for the *E. coli* bacteria, showing that from

the 4th week postpartum onwards this bacterium does not have a frequency as high as at the beginning of the puerperium.

Among SE cows, there was no *T. pyogenes* isolation, corroborating MADOZ et al. (2013) findings that did not identify *T. pyogenes* in uterine samples from cows diagnosed with SE in Argentina. Moreover, SENS and HEUWIESER (2013) highlighted that *T. pyogenes* does not seem to be the main key for cows to develop subclinical endometritis. Our observations corroborate previous studies (WANG et al., 2018), supporting that uterine infections with the main pathogens play a minor role in cows with SE compared to CE cows. The uterine defense mechanism that occurs while the inflammatory response is still in progress can remove the intrauterine bacteria, restoring the normal uterine environment (APPIAH et al., 2020). The establishment of uterine infections depends on the immune status of the animal and the pathogenicity of the invading microorganisms (WANG et al., 2018). Several factors can influence the pathogenicity of the microorganisms, including the bacterial load, strain virulence and the interactions between species.

Our findings also revealed that *Prevotella heparinolytica*, *Porphyromonas levii*, *Fusobacterium necrophorum*, *Bacteroides pyogenes*, and *Helcococcus* spp. were identified only in CE cows. In dairy herds in Canada, PASCOTINI et al. (2020) identified, at the genus level, that cows with CE had a higher prevalence of *Helcococcus*, *Fusobacterium*, *Trueperella*, and *Porphyromonas*, and *Fusobacterium* and *Trueperella* than healthy and SE cows, respectively. In a commercial dairy farm in China, WANG et al. (2018) highlighted a positive correlation between *Fusobacterium*, *Bacteroides*, *Porphyromonas*, and *Helcococcus*, in addition to *Trueperella*, with CE in dairy cows compared to healthy and SE cows.

In the present study, the methods of sample collection, transportation, and culture media used permitted the isolation of a large diversity of aerobic and anaerobic agents, besides the limitations of culture-dependent techniques. PASCOTINI et al. (2020) described that the isolation of anaerobic agents was reduced when compared with the abundance observed in the metagenomic analysis. The authors describe that the number of samples collected and more controls in anaerobic culture could be important in their study. In our study it was demonstrated that the technique used for culture and identification of microorganisms present in the uterine environment made it possible to isolate a large diversity of bacterial species, aerobic and anaerobic.

We highlight that 18.1% and 40% of the samples from CE and SE cows, respectively, did not present bacterial isolation. These results emphasize the importance of identifying the microorganisms in the microbiota of dairy cows affected by uterine diseases for the real

knowledge of the main microorganisms associated with uterine diseases that can contribute to farm decision making.

5.6 CONCLUSION

This study provides an understanding of the frequently isolated uterine microorganisms in dairy cows during the puerperal period. Our results provide important insights into the identification of the diverse and complex microorganisms community. The identification of bacterial microorganisms present in the uterine microbiota of dairy cows during early postpartum phase may serve as a basis for future strategies for uterine therapies, enabling the optimization of the decision-making on strategic treatments in dairy farms, favoring the rational use of antibiotics in dairy cows. In addition, MALDI-TOF MS-based identification provides less expensive and faster bacterial species identification than conventional phenotypic identification methods. This is especially relevant for research related to uterine diseases and veterinarians and technicians, as it allows quick access to the knowledge of the microorganisms that are affecting animals with uterine diseases, contributing to the early treatment of dairy cows.

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STUDY 2: ASSESSMENT OF CLINICAL AND SUBCLINICAL ENDOMETRITIS IMPACTS ON THE REPRODUCTIVE PERFORMANCE AND MILK PRODUCTION OF DAIRY COWS

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6 STUDY 2: ASSESSMENT OF CLINICAL AND SUBCLINICAL ENDOMETRITIS IMPACTS ON THE REPRODUCTIVE PERFORMANCE AND MILK PRODUCTION OF DAIRY COWS

6.1 ABSTRACT

The aim of this study was to investigate the impacts of clinical endometritis (CE) and subclinical endometritis (SE) on the reproductive performance and milk production of dairy cows reared in tropical conditions. A total of 279 lactating Holstein dairy cows (28 to 33 d in milk) from six commercial farms were studied. These were classified in three groups: healthy cows (without CE and SE, n = 161), cows with CE (vaginal discharge score = 3 and ≥ 18% PMNL, n = 83) and cows with SE (absence of signs of CE and > 18% PMNL, n = 35). Cows with CE had a lower conception rate at first AI ($P < 0.05$). Furthermore, both cows with CE and SE needed more service to become pregnant and days to be pregnant ($P < 0.05$). Cows with CE and SE produced less milk than healthy cows ($P < 0.05$). The logistic regression showed that any factor evaluated in this study were not associated with the occurrence of CE and SE. In conclusion, our results reveal a negative impact of CE and SE on the reproductive performance and milk production of dairy cows.

Keywords: clinical endometritis, subclinical endometritis, reproductive outcome, dairy cattle

6.2 INTRODUCTION

Uterine diseases have a negative impact on the economy of dairy farms, mainly due to expenses with the treatment and disposal of milk due to the use of certain antibiotics, and reduced milk production of the affected animals (LEBLANC, 2008; HAIMERL et al., 2017; PAIANO et al., 2021a). Furthermore, cows with uterine diseases may show a worsening of reproductive performance including delay in the resumption of ovarian activity after calving, reduction in conception and pregnancy rates and increase in the calving-to-conception interval, which can cause an increase in the involuntary culling of these animals (LEBLANC et al., 2002; ŠAVC et al., 2016; LIMA et al., 2019; PAIANO et al., 2020a).

Clinical endometritis (CE) and subclinical endometritis (SE) are common uterine diseases in dairy cows (SHELDON et al., 2006). The CE is characterized as an inflammation of the endometrium with the presence of purulent or mucopurulent uterine secretion at 21 or more days postpartum (LEBLANC et al., 2002). While SE is characterized by an increase in the proportion of polymorphonuclear neutrophils leukocyte (PMNL) in the endometrium, with no clinical signs of CE (KASIMANICKAM et al., 2004). The main risk factors for uterine diseases in dairy cows are abortions, stillbirths, dystocia and retained placenta (PRUNNER et al., 2014; ADNANE et al., 2017).

Previous studies carried out on dairy cattle herds in North America (RIBEIRO et al., 2013; BICALHO et al., 2016;) and Europe (LAMBERTZ et al., 2014; CANADAS et al., 2020) reported reduced reproductive performance of cows affected by endometritis. However, in Brazil, to the best of our knowledge, there are no studies describing reproductive performance and risk factors of cows without uterine disease and cows affected by CE and SE between 28 and 33 days after calving.

The knowledge of reproductive performance and milk production of cows with uterine diseases and the factors associated with the occurrence of endometritis of dairy cows is essential to avoid financial losses caused by these diseases to the health of herds. In view of the damage caused by uterine diseases in animal health, strategies are necessary for their early diagnosis and treatment. Given this impetus, the objectives of this study were to assess the main factors associated with the occurrence of CE and SE and to characterize the impacts of CE and SE on the reproductive performance and milk production of dairy cows reared in tropical conditions in southeastern Brazil.

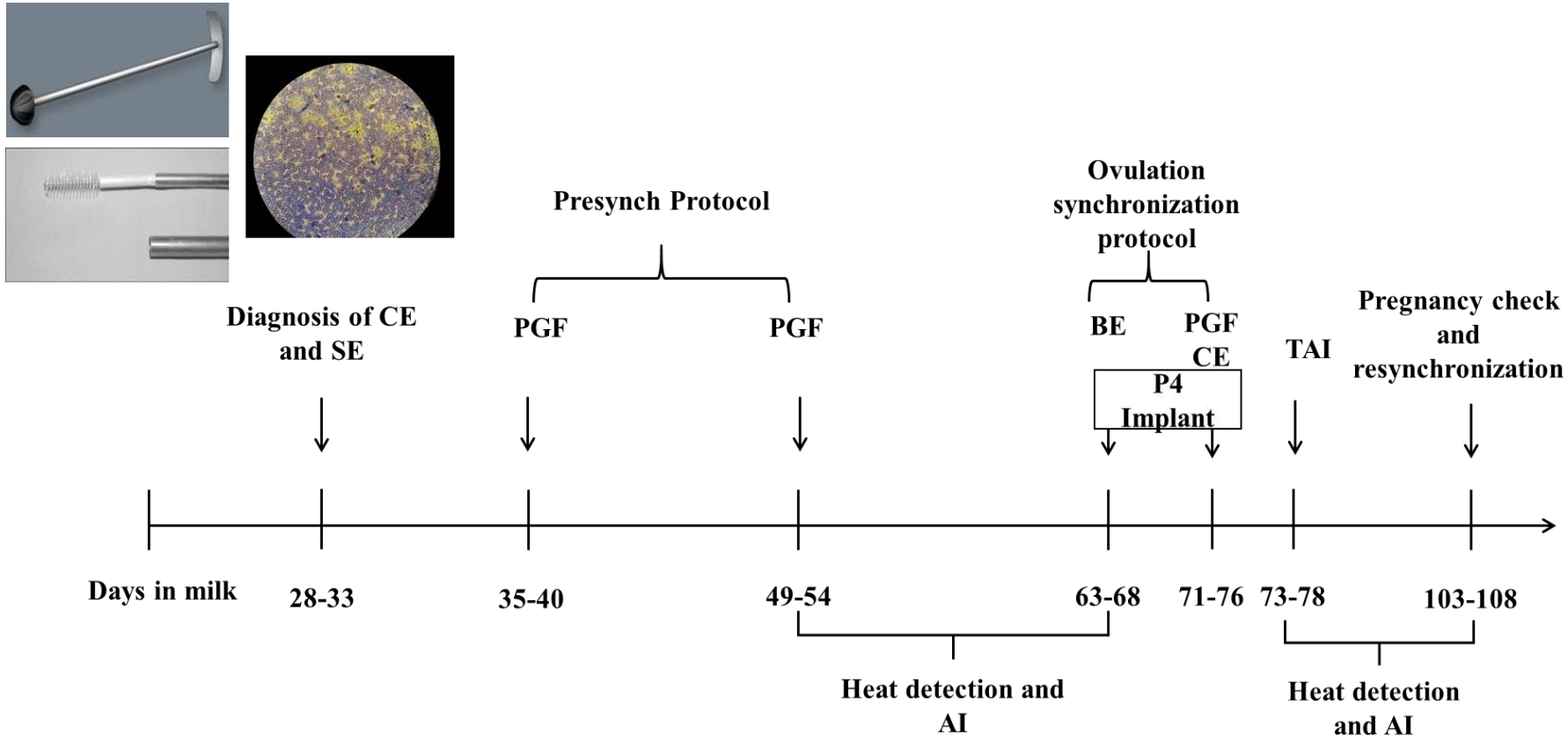
6.3 MATHERIALS AND METHODS

6.3.1 Study farms

Six commercial dairy farms in São Paulo State, Brazil, were evaluated between May and November of 2019. At the time of the study, the farms consisted of 220 (Farm 1), 240 (Farm 2), 120 (Farm 3), 115 (Farm 4), 105 (Farm 5) and 90 (Farm 6) lactating Holstein cows and the rolling herd average milk production (305-day) ranged from 8,200 to 8,600 kg. Lactating dairy cows were housed in freestall barns, with concrete stalls covered with sand beds. Cows were fed twice daily with diet of total mixed ration (TMR) consisting of corn silage, with corn meal, soybean meal and mineral supplements, formulated according to NRC (2001) to meet the nutritional requirements for lactating Holstein cows. Cows were milked two times daily.

Reproductive management used a combination of detection of estrus and Presynch and artificial insemination (AI) (MOREIRA et al., 2001) (Figure 10). For the first service, all cows were inseminated by timed AI following completion of the Presynch protocol. Pre-synchronization was performed using two injections of $PGF_{2\alpha}$ (cloprostenol; Sincrocio, OuroFino Animal Health, Ribeirão Preto, Brazil, 530 μg, i.m.), separated by 14 days. Cows were synchronized for TAI protocol using 2 mg (i.m.) of estradiol benzoate (Sincrodiol; OuroFino Animal Health, Ribeirão Preto, Brazil) and progesterone (P4) intravaginal implant insert containing 1.0 g of P4 (Sincrogest; OuroFino Animal Health, Ribeirão Preto, Brazil) on day −10; $PGF_{2\alpha}$ (cloprostenol; Sincrocio, OuroFino Animal Health, Ribeirão Preto, Brazil, 530 μg, i.m.), P4 implant removal and treatment with 1.0 mg of estradiol cypionate (E.C.P.; Zoetis, São Paulo, Brazil) on day −2; and TAI on day 0. Cows observed in natural estrus or after estrus synchronization using PGF2α were artificially inseminated according to the a.m. and p.m. rule.

Figure 10. Diagram of activities during the study.



Fonte: Paiano, R. B. (2021).

6.3.2 Study design

Two hundred and seventy-nine Holstein dairy cows were sampled in this longitudinal study. All cows (n = 279) were examined between 28 and 33 days after parturition. Only cows that have not been treated with antibiotics or non-steroidal or steroidal anti-inflammatory drugs after parturition and were not diagnosed with mastitis, hypocalcemia and ketosis were included in this study. The study was approved by the Bioethics Committee of the University of São Paulo (Protocol No. 1377270218).

6.3.3 Animal classification

The vaginal discharge was obtained using a Metricheck (SimcroTech, Hamilton, New Zealand) as previously described (WILLIAMS et al., 2005). Cows with the score 3 (> 50% of purulent vaginal discharge) were considered to have CE. The SE was diagnosed by endometrial cytology using the cytobrush technique (KASIMANICKAM et al., 2004).

Briefly, a sterile cytobrush (Disposable cytology sampling brush 8”; Viamed Ltd, West Yorkshire, UK) was attached to the tip of a conventional artificial insemination (AI) gun, covered by a disposable AI sheath and protected by a sanitary sheath, to prevent contamination. After cleaning the vulva with a paper towel, disinfected with 70% ethanol, the cytobrush was inserted under transrectal control through the vagina, into the uterine body. Inside the uterus, the sleeve was retracted and the brush pushed gently into the lumen. The brush was pushed gently forward until emerging out of the apparatus. The cells were collected by rotating the brush on the dorsal wall of the uterus, and then reintroduced back in the protective sheath of the gun during the passage through the genital tract.

Outside the cow, the cytobrush was removed from the pistol grip and rotated on a microscopic slide, and stained with panoptic rapid staining method (Laborclin ®, Pinhais, Brazil). Evaluations were performed under a microscope at 400× magnification (Nikon, E200, Tokyo, Japan). A total of 200 cells were counted in the cytological slide to determine the proportion of PMNL. The threshold value for the proportion of PMNL cells indicating SE was set at 18% (KASIMANICKAM et al., 2004). Thus, based on uterine health, three groups of dairy cows were formed: healthy cows (without CE and SE, n = 161), cows with CE (vaginal discharge score = 3 and > 18% PMNL, n = 83) and cows with SE (absence of signs of CE and

> 18% PMNL, n = 35). Cows that had a vaginal discharge score = 3 and PMNL < 18 were not included in the study.

6.3.4 Statistical analysis

Reproductive performance data (days to first AI, conception at first AI, days to pregnancy and number of services per pregnancy) and the milk production (recorded on the day of sampling) were obtained from the herd's management software. Reproductive performance was characterized by median days to first AI (number of days from parturition to first service), conception at first AI (number of cows pregnant after first AI divided by number of cows inseminated × 100), median days to pregnancy (number of days from parturition to pregnancy), and services per pregnancy (total number of inseminations divided by the number of pregnant cows).

Continuous variables were analyzed by linear regression and ANOVA and binary variables were analyzed by logistic regression using the GLIMMIX procedure (version 9.4; SAS/STAT; SAS Institute Inc., Cary, NC). The interval to an event was analyzed by the Cox's proportional hazard model, using the PHREG procedure of SAS. The median days to an event was determined by undertaking survival analysis using the Kaplan-Meier model and the LIFETEST procedure within SAS software. Values were censored when the observations were terminated for reasons beyond the control of the investigator, such as when cows were not pregnant at the end of the study period or culled from the herd during the study period before becoming pregnant. The models included the fixed effect of the experimental group (cows with CE, SE and healthy cows) and the random effects of parity and farm. Statistical significance was set at $P < 0.05$.

The risk factors for CE and SE were analyzed by logistic regression using the LOGISTIC procedure of SAS software. Included factors were: CE, SE, parity (1, 2 and ≥ 3), body condition score (normal = 3.0-3.25 points, thin = ≤ 2.75 points and fat = ≥ 3.50 points) and milk production (medium = 26-29.9 kg, low = ≤ 25.9 kg and high = ≥ 30 kg). The model produced odds ratios (OR) as estimates of the strength of association between the potential risk factors and CE and SE.

6.4 RESULTS

No difference was observed between the occurrence of CE (Farm 1: 31.3% 30/96; Farm 2: 27.1% 13/48; Farm 3: 28.2% 11/39; Farm 4: 28.6% 10/35; Farm 5: 31.3% 10/32; Farm 6: 31.0% 9/29) and SE (Farm 1: 11.5% 11/96; Farm 2: 12.5% 6/48; Farm 3: 15.4% 6/39; Farm 4: 11.4% 4/35; Farm 5: 12.5% 4/32; Farm 6: 13.8% 4/29) among the sampled herds.

The CE cows had a 57.2% decrease in conception in the first AI in conception at first AI ($P = 0.0002$), required 1.4 more services per conception ($P < 0.001$) and had a milk production reduced by 4.4 kg than healthy cows ($P < 0.001$; Table 4). While cows with SE had a 34.3% decrease in conception AI ($P < 0.001$), required 1.1 more services per conception ($P < 0.001$) and had a milk production reduced by 4.5 kg than healthy cows ($P < 0.001$; Table 4).

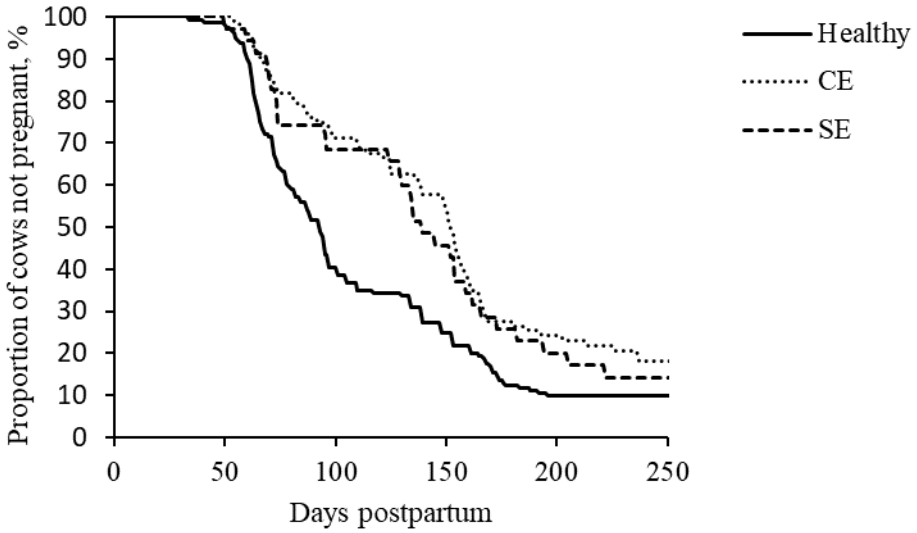
Table 4. Reproductive performance and milk production of healthy, clinical (CE) and subclinical (SE) endometritis in dairy cows.

Item	**CE**	**SE**	**Healthy**	***P*-value**
Number of cows	83	35	161	
Days to first AI	73.4 ± 3.5	71.7 ± 3.1	68.9 ± 3.9	0.5930
Conception rate at first AI (%)	20.5[b]	31.4[b]	47.8[a]	0.0002
Services per pregnancy	3.1 ± 0.4[a]	2.8 ± 0.2[a]	1.7 ± 0.3[b]	< 0.001
Milk production (kg)	23.8±0.57[b]	23.7±0.88[b]	28.2±0.40[a]	< 0.001

[a,b] Values within a row with different superscript letters differ at $P < 0.05$.

Kaplan-Meier survival analysis (Figure 11) revealed that CE and SE group had 50.3 and 43.1 days longer ($P < 0.001$), respectively, than the healthy group.

Figure 11. Kaplan-Meier survival curves for time to pregnancy up to 250 d postpartum in the CE (n = 83), SE (n = 35) and healthy (n = 161) groups. The percentage of censored cows was 10.6%, 18.1% and 14.3% for healthy, clinical endometritis (CE) and subclinical endometritis (SE), respectively. The probability of pregnancy by 250 days postpartum was higher in the healthy group (93.6 d) than in the CE (143.9 d; hazard ratio = 3.61; confidence interval = 2.726–5.319; $P < 0.0001$) and SE (136.7 d; hazard ratio = 2.919; confidence interval = 1.925–4.425; $P < 0.0001$) group.



Tables 5 and 6 show the results of the logistic regression for the risk factors associated with the occurrence of CE and SE, respectively. The occurrence of CE and SE was not associated with any factor evaluated in this study.

Table 5. Results of univariable model of risk factors associated with of clinical endometritis (CE) at the day of enrollment.

Factor	%	no./no.	OR	95.0% CI	*P*-value
Parity					
≥3	29.2	26/89	Reference	-	0.4891
1	36.7	29/79	1.405	0.734-2.692	
2	36.8	28/76	1.413	0.734-2.724	
BCS[1]					
Fat	33.1	40/121	Reference	-	0.6816
Thin	37.7	29/77	1.223	0.674-2.222	
Normal	30.4	14/46	0.886	0.425-1.845	
Milk production[2]					
Medium	32.9	27/82	Reference	-	0.3462
High	25.6	21/82	0.843	0.433-1.640	
Low	43.8	35/80	1.358	0.712-2.589	

[1] Normal = 3.0-3.25 points of BCS, thin = ≤ 2.75 points of BCS and fat = ≥ 3.50 points of BCS.

[2] Medium = 26-29.9 kg, low = ≤ 25.9 kg and High = ≥ 30 kg.

Table 6. Results of univariable model of risk factors associated with of subclinical endometritis (SE) at the day of enrollment.

Factor	%	no./no.	OR	95.0% CI	*P*-value
Parity					
≥3	13.7	10/73	Reference	-	0.4108
1	18	11/61	1.386	0.545-3.525	
2	22.6	14/62	1.837	0.751-4.493	
BCS[1]					
Fat	19	19/100	Reference	-	0.7472
Thin	18.6	11/59	0.977	0.429-2.227	
Normal	13.5	5/37	0.666	0.229-1.936	
Milk production[2]					
Medium	19.1	13/68	Reference	-	0.4869
High	13.4	9/67	0.656	0.258-1.668	
Low	21.3	13/61	1.146	0.482-2.725	

[1] Normal = 3.0-3.25 points of BCS, thin = ≤ 2.75 points of BCS and fat = ≥ 3.50 points of BCS.

[2] Medium = 26-29.9 kg, low = ≤ 25.9 kg and High = ≥ 30 kg.

6.5 DISCUSSION

Information on reproductive performance and factors associated with the occurrence of uterine diseases of dairy cows are essential for veterinarians, technicians and producers to understand the impact of these diseases on herd fertility. However, there is a lack of information for dairy herds reared in tropical conditions. Dairy farms located in tropical conditions are characterized by high pluviometric indices and high temperatures, favoring the growth of pasture and low-cost milk production (BARUSELLI et al., 2004). However, cows in tropical conditions may have a higher incidence of postpartum anestrus than cows bred in subtropical conditions, which contributes to the worsening of reproductive performance, resulting in a longer interval between parturition to conception (BARUSELLI et al., 2004).

Results from the current study indicate negative impact of CE and SE on reproductive outcomes. Endometritis causes severe economic losses associated with the negative impact on reproductive performance, which are estimated to be € 292 per case (OPSOMER, 2015). In addition, a recently published study showed that dairy cows affected by endometritis associated with *Trueperella pyogenes* had a worsening in reproductive performance and milk production when compared to cows without uterine disease (PAIANO et al., 2021b).

Previous studies have observed that dairy cows with CE and SE had delayed a time to pregnancy and lower pregnancy per artificial insemination than healthy group (KASIMANICKAM et al., 2004; MADOZ et al., 2008; WERNER et al., 2012), in agreement with the data of the present study. PAIANO et al. (2019) reported 34% decrease in the conception rate at first AI and a 1.1 increase in the number of services per pregnancies in cows with CE than in control cows. Cows with CE presented an increase of 57 and 79 days to become pregnant than cows without CE in dairy herds in United States (BICALHO et al., 2016) and Argentina (GIULIODORI et al., 2013), respectively. In Spain, SE increased 35 days in the interval between calving to pregnancy and increased 1.0 the service per pregnancy when compared to cows without SE (BARRIO et al., 2015). MADOZ et al. (2013) found that cows with SE had 16% reduction in the pregnancy rate and had 30 days more to get pregnant than cows without SE in dairy herds in Argentina.

Regarding the milk production, CE and SE groups had a lower milk production when compared to healthy cows. This lower production represents a reduction of more than 4 kg of milk per cow per day. The results of the present study are in agreement with PAIANO et al. (2019), who reported a lower milk production in cows with CE when compared with cows without CE. This lower production may be caused due to inflammation of the uterine

epithelium, which may be associated with a greater release of inflammatory cytokines into the bloodstream (SHELDON and OWENS, 2017). In addition, the pain related to pro-inflammatory status can reduce food intake and consequently cause reduction in milk production. Furthermore, previous health problems such as subclinical hypocalcemia and subclinical ketosis can increase the risk of cows developing puerperal diseases, which may result in less milk production (PAIANO et al., 2020b).

According to our results, no evaluated factors were associated with an increased occurrence of CE and SE. Corroborating with these data, PLÖNTZKE et al. (2010) and PASCOTTINI et al. (2017) also did not found an association with the same factors evaluated in the present study and CE and SE. These association depend mainly on geographic region and herd and nutritional management (GIULIODORI et al., 2017). It can be speculated whether additional effects could be due to factors not evaluated in this study, such as reduced immunity, food quality, environmental conditions such as excess moisture in bed, frequency of changing the bed, unhygienic parturition stall and social factors like overcrowding, hierarchy and dominance within the herd that can limit food intake. All of these factors can influence the occurrence of uterine diseases on farms.

6.6 CONCLUSION

The negative impact of CE and SE on the reproductive performance and milk production of cows reared in tropical conditions herds were undeniable. The results of the current study demonstrate that both cows in the CE and SE groups had a reduction in the conception rate at the first service and required more services to obtain pregnancy, in addition to having lower milk production when compared to cows without uterine disease.

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STUDY 3: CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS AGAINST PATHOGENS OFTEN RELATED TO CATTLE ENDOMETRITIS

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Appendix A

7 STUDY 3: CHEMICAL COMPOSITION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS AGAINST PATHOGENS OFTEN RELATED TO CATTLE ENDOMETRITIS

7.1 ABSTRACT

Introduction: Endometritis is a condition marked by inflammation of the endometrium that affects dairy cows from 21 days after parturition, causing damage to herd fertility and economic losses on farms. The use of active compounds obtained from plant sources has gained importance as disease treatment agents in farm animals due to the high resistance rates currently observed against traditional antibiotics commonly used. The study was carried out to examine the chemical composition and to investigate the antibacterial activity of rosemary, cinnamon, cloves, eucalyptus, lemon, oregano and thyme essential oils against the reference strain of *Escherichia coli* (ATCC 25922), *Fusobacterium necrophorum* (ATCC 25286), *Trueperella pyogenes* (ATCC 19411) and *Staphylococcus aureus* (ATCC 29213), considered as typical bacteria causing endometritis.

Methodology: The chemical composition of the seven essential oils were analyzed by GC-MS and their antibacterial activity was evaluated by the disk diffusion method.

Results: Thirty-six components were identified in total using GC-MS analyzes. The main compounds were cinnamaldehyde (86.5% for cinnamon essential oil), eugenol (85.7% for clove essential oil), 1,8-cineol (80% for eucalyptus and 47.8% rosemary essential oils), limonene (65.5% for lemon essential oil), carvacrol (72.1% for oregano essential oil) and thymol (48.8% for thyme essential oil). The disk diffusion assay revealed that cinnamon, clove, oregano, and thyme essential oils showed the best results compared to the other three essential oils, showing the largest zone of inhibition against all bacteria evaluated.

Conclusions: These findings indicated that essential oils are a potential agent to be used as an alternative for bovine endometritis treatment.

Keywords: Bovine endometritis; essential oils; antimicrobial activity; phytotherapy.

7.2 INTRODUCTION

Uterine diseases cause profound economic losses in the dairy sector, mainly due to costs related to decreased milk production, increased use of medicines to treat diseases, discarding milk through antibiotics, and the damage caused by death or early culling of the cows (LEBLANC, 2008, HAIMERL et al., 2017). Among uterine diseases, endometritis is one of the most important, being characterized by inflammation of the endometrium from 21 days after parturition (LEBLANC et al., 2002; GILBERT et al., 2005; PAIANO et al., 2019a), with purulent or mucopurulent uterine discharge (PAIANO et al., 2019b).

The prevalence of endometritis reported in Brazil was 28.4% in 338 cows evaluated (PAIANO et al., 2019b). The bacteria most often described causing endometritis are *Trueperella pyogenes*, *Escherichia coli* and *Fusobacterium necrophorum* (LEBLANC, 2008; SHELDON et al., 2008). The use of antibiotics is the most used therapy against endometritis (LEBLANC, 2008). However, indiscriminate use of antibiotics may contribute to increased resistance of pathogenic bacteria, compromising the success of therapy, and may cause low efficacy of the drugs (LIU et al., 2009).

In this sense, the use of products of natural origin has become an alternative to reduce the use of antibiotics in dairy cows. Thus, essential oils are volatile substances naturally produced by plants as secondary metabolites, and are known for their antibacterial, antifungal, and antiviral properties, among others (BONILLA and SOBRAL, 2019). They can be extracted from various parts of plants, such as roots, leaves, bark, seeds, and fruits (BAKKALI et al., 2008; SZWEDA et al., 2018). Their components include two classes of separate biological origin: the prime group consists of terpenes and terpenoids, and the second of aliphatic and aromatic components (TARIQ et al., 2019). According to PAULI and SCHILCHER, (2010), the antimicrobial activity of essential oils can be witnessed by *in vitro* tests, being the most three important ones are the agar diffusion, the agar or broth dilution and the vapor phase test.

The use of essential oils in cattle has increased in recent years, and the action in the treatment of diarrhea in calves (KATSOULOS et al., 2017) and mastitis (SZWEDA et al., 2018) has been reported. However, there is little information on the use of essential oils as a natural therapy for endometritis. In this context, the aim of the present study were to characterize the chemical composition and to investigate the antibacterial properties of rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum cassia*), clove (*Eugenia caryophyllus*), eucalyptus

(*Eucalyptus globulus*), lemon (*Citrus limon*), oregano (*Origanum vulgare*) and white thyme (*Thymus vulgaris*) essential oils against four bacteria' strains causing endometritis.

7.3 METHODOLOGY

7.3.1 Essential oils

The essential oils of rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum cassia*), clove (*Eugenia caryophyllus*), eucalyptus (*Eucalyptus globulus*), lemon (*Citrus limon*), oregano (*Origanum vulgare*) and white thyme (*Thymus vulgaris*) were obtained from Ferquimica® (Vargem Grande Paulista, São Paulo, Brazil).

7.3.2 Gas chromatography/mass spectrometry (GC-MS) analysis

The essential oils chemical components were identified by gas chromatograph coupled to mass spectrometry (GC-MS). GC analyses were performed using a Shimadzu GC-2010 gas chromatograph, equipped with a GCMS-QP2010 Ultra mass spectrometer (Shimadzu, Suzhou, China). A split/splitless injector was used. Sample (1 μl) was injected into the injector with a split ratio of 1:10. Oven temperature was 40 ^{o}C for 3 min, then programmed heating from 40 to 280 ^{o}C at a rate of 8 ^{o}C/min. Injector temperature was 250 ^{o}C. Helium was used as carrier gas with 14 mL/minute flow rate. The volatile compounds were identified by comparison with mass spectra with those recorded in the National Institute of Standards and Technology database.

7.3.3 Bacterial strains

The evaluated bacterial strains in this study were *Escherichia coli* (ATCC 25922), *Fusobacterium necrophorum* (ATCC 25286), *Trueperella pyogenes* (ATCC 19411) and *Staphylococcus aureus* (ATCC 25923). All microorganisms were cultured in BHI broth (Brain Hearth Infusion, Acumedia, Lansing, MI, USA), being incubated at 37 ^{o}C for 24 hours (*E. col*i and *S. aureus* strains) or 48 hours (*T. pyogenes* and *F. necrophorum* strains).

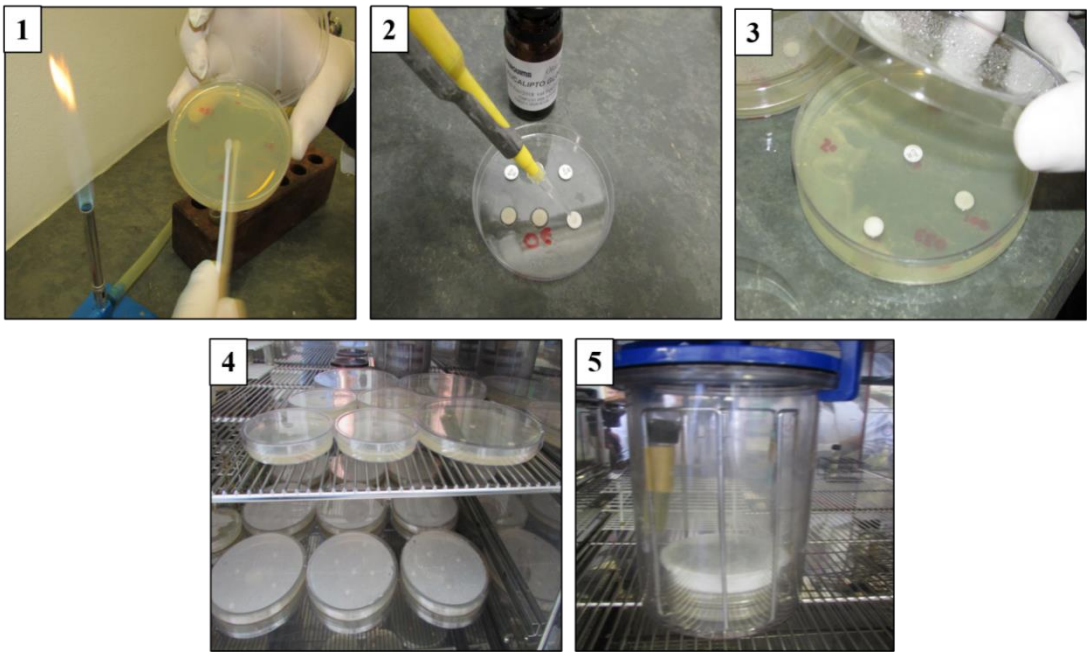
7.3.4 Disk diffusion assay

After incubation period the cultures were diluted in sterile saline solution and the turbidity adjusted to the 0.5 standard McFarland scale (~10^8 CFU/mL). With the use of a sterile cotton swab, surface of plates containing Mueller-Hinton agar (MHA; Difco) were inoculated with the bacterial suspension (Figure 12). To test *T. pyogenes* strain, the MHA was supplemented with 5% sheep blood and to test *F. necrophorum* strain, the medium used was *Brucella* agar (Acumedia, Lansing, MI, USA), supplemented with 5% sheep blood, hemine (Interlab Difco, Sao Paulo, Brazil) and vitamin K1 (Interlab Difco, São Paulo, Brazil).

Paper disks with 6 mm diameter (Whatman no 3) soaked with 20 µL of each pure essential oil (Figure 12) were laid on the surface of inoculated agar (Figure 12). Discs of ceftiofur (30 µg, Cefar Diagnósticos Ltda., São Paulo, Brazil) were used as positive control. A paper disc soaked with 20 µL of solution consisting of phosphate buffered saline (PBS, Sigma, São Paulo, Brazil) with 0.5% (v/v) polysorbate 80 (Tween 80) was used as negative control and loaded in each tested plate.

The plates were incubated at 37 °C for 24 hours (*E. coli* and *S. aureus*) or 48 hours (*T. pyogenes*) in aerobic conditions (Figure 12), or 37 °C for 48 hours in anaerobic condition (*F. necrophorum*). Anaerobic conditions were maintained by using an anaerobic jar with anaerobic atmosphere generator (Anaerobac, Probac, São Paulo, Brazil) (Figure 12). After incubation, the inhibition zone diameter (IZD) was measured in accordance with the CLINICAL AND LABORATORY STANDARDS INSTITUTE guidelines (CLSI, 2018) and all experiments were carried out in three independent replicates.

Figure 12. Scheme of disk diffusion assay.



(1) Inoculation of bacteria on the plates with the agar. (2) Impregnation of paper discs with 20 µL of pure essential oil. (3) Plate inoculated with the bacteria containing the discs of the seven essential oils, positive and negative controls. (4) Incubation of bacteria in aerobic conditions. (5) Incubation of bacteria in anaerobic condition.

Fonte: Paiano, R. B. (2021).

7.3.5 Statistical analysis

Data were subjected to analysis of variance (ANOVA) and the differences between the means and standard error were tested by Tukey test. Statistical significance is considered as $P < 0.05$.

7.4 RESULTS

7.4.1 Chemical composition of the essential oils

The volatile compounds for all studied essential oils are listed in Table 7. The major chemical constituent found in cinnamon essential oil was cinnamaldehyde (86.5%), and in clove essential oil was eugenol (85.7%). The eucalyptus essential oil was particularly rich in 1,8-cineol (80.0%), while the essential oil of lemon contained a high percentage of limonene (65.5%). In oregano essential oil the most abundant compound was carvacrol (72.1%), and in rosemary essential oil was 1,8-cineol (47.8%). Thymol (48.8%) and p-cymene (26.4%) were the main compounds identified in the thyme essential oil.

Table 7. Chemical composition (%) for seven essential oils obtained with GS-MS analysis.

Compounds	**Cinnamon**	**Clove**	**Eucalyptus**	**Lemon**	**Oregano**	**Rosemary**	**Thyme**
Benzaldehyde	2.40	-	-	-	-	-	-
Borneol	0.95	-	-	-	0.90	2.70	0.33
Bornyl acetate	-	-	-	-	-	0.90	-
δ-Cadinene	-	0.10	-	-	-	-	-
Camphene	-	-	-	-	-	4.50	0.92
Camphor	-	-	-	-	-	11.90	-
Carvacrol	-	-	-	-	72.12	-	2.88
α-Caryophyllene	-	1.81	-	-	-	-	0.11
β-Caryophyllene	-	11.50	-	-	3.03	3.50	1.21
Caryophyllene oxide	-	0.17	-	-	-	-	0.08
1,8-Cineol	-	-	80.04	-	-	47.80	-
p-Cymene	-	-	2.96	-	4.81	1.40	26.43
Cinnamaldehyde	86.50	-	-	-	-	-	-
Cinnamyl alcohol	0.91	-	-	-	-	-	-
Coumarin	2.11	-	-	-	-	-	-
Decanol	-	-	-	0.04	-	-	-
α-Farnesene	-	0.08	-	-	-	-	-
Eugenol	-	85.73	-	-	-	-	-
Geranyl acetate	-	-	-	0.12	-	-	-
α-Humulene	-	-	-	-	1.01	-	-

Isoborneol	-	-	-	-	-	-	0.29
Limonene	-	-	9.02	65.59	0.83	2.20	1.05
Linalool	-	-	-	0.13	3.03	-	4.51
Myrcene	-	-	-	1.55	-	1.50	1.04
Neral	-	-	-	0.60	-	-	-
Neryl acetate	-	-	-	0.21	-	-	-
α-Pinene	-	-	3.97	2.34	0.30	11.40	3.43
β-Pinene	-	-	-	15.06	-	7.80	0.54
Sabinene	-	-	-	1.76	-	0.10	-
Salicylaldehyde	1.85	-	-	-	-	-	-
Styrene	2.34	-	-	-	-	-	-
α-Terpineol	-	-	-	-	0.70	1.70	0.48
γ-Terpinene	-	-	4.01	7.93	4.81	0.70	6.05
γ-Terpineol	-	-	-	-	-	-	0.14
Terpinen-4-ol	-	-	-	-	0.83	0.90	-
Thymol	-	-	-	-	2.04	-	48.80

7.4.2 Antibacterial activity

The *in vitro* antibacterial activities of seven essential oils against several bacteria strains were qualitatively and quantitatively assessed by the measuring of IZD using the agar disk diffusion method as shown in Table 8.

Table 8. Inhibition zone diameter identified by disk diffusion method with essential oils tested.

	IZD - Inhibition zone diameter (mm)[a]								
Bacteria	**Cinnamon**	**Clove**	**Eucalyptus**	**Lemon**	**Oregano**	**Rosemary**	**Thyme**	**Positive control**	**Negative control**
Escherichia coli ATCC 25922	32.33±1.29^{a}	9.67±1.29^{c}	8.33±1.29^{c}	6.00±1.29^{c}	20.67±1.29^{b}	8.00±1.29^{c}	26.67±1.29^{a}	27.86±1.29^{a}	6.00±1.29^{c}
Trueperella pyogenes ATCC 19411	29.67±0.31^{a}	15.00±0.31^{d}	6.00±0.31^{e}	6.00±0.31^{e}	21.00±0.31^{c}	6.00±0.31^{e}	25.00±0.31^{b}	21.33±0.31^{c}	6.00±0.31^{e}
Fusobacterium necrophorum ATCC 25286	32.00±1.13^{a}	21.67±1.13^{b}	6.00±1.13^{c}	6.00±1.13^{c}	22.00±1.13^{b}	6.00±1.13^{c}	24.67±1.13^{b}	37.33±1.13^{a}	6.00±1.13^{c}
Staphylococcus aureus ATCC 25923	38.33±0.75^{a}	15.33±0.75^{c}	8.00±0.75^{d}	6.00±0.75^{e}	36.00±0.75^{a}	10.67±0.75^{b}	36.00±0.75^{a}	29.00±0.75^{b}	6.00±0.75^{e}

[a] Inhibition zone diameter, values represent mean of three replicates ± standard error. Different letters in the same line represent statistical difference ($P < 0.05$) in the size of inhibition zones including diameter of disc 6 mm formed under the paper disc by each essential oil.

The results obtained with ceftiofur against *E. coli* ATCC 25922 (27.86 mm of IZD) and *S. aureus* ATCC 25923 (29.00 mm of IZD) strains were within the expected values according to (CLSI, 2018) (Table 8). These results revealed that the cinnamon oil presented the greater IZD that varied from 29.67 to 38.33 mm. The larger IZD was observed in *S. aureus* (38.33 mm), and smaller IZD was observed in *T. pyogenes* (29.67 mm). Clove essential oil produced an IZD varying from 9.67 to 21.67 mm, being the smaller IZD observed against *E. coli* (9.67 mm) and the larger IZD observed against *F. necrophorum* (21.67 mm). Eucalyptus essential oil produced an IZD varying from 8 to 8.33 mm, the smaller IZD was observed against *S. aureus* (8 mm) and the larger IZD against *E. coli* (8.33 mm) and no effect were observed on *F. necrophorum* and *T. pyogenes*. The lemon essential oil presented no IZD against any strains tested. The IZD produced by oregano essential oil varied from 20.67 to 36 mm, being the smaller IZD observed against *E. coli* (20.67 mm) and the larger IZD showed against *S. aureus* (36 mm). Rosemary essential oil produced an IZD varying from 8 to 10.67 mm, the smaller IZD was observed against *E. coli* (8 mm) and the larger IZD was seen against S. aureus (10.67 mm) with no inhibition effects being observed against *F. necrophorum* and *T. pyogenes*. The inhibition zone produced by the thyme essential oil varied from 24.67 to 36 mm, the smaller IZD was observed against *F. necrophorum* (24.67 mm), whereas the larger IZD was seen showed against *S. aureus* (36 mm).

For *E. coli*, essential oil of cinnamon and thyme had the larger ($P < 0.05$) IZD compared to the other essential oils. For *T. pyogenes* and *F. necrophorum*, cinnamon essential oil had the larger ($P < 0.05$) IZD compared to the other essential oils. Against the *S. aureus*, essential oil of cinnamon, oregano and thyme had the highest ($P < 0.05$) IZD compared to the other essential oils and positive control.

7.5 DISCUSSION

GOÑI et al., (2009) showed that the major components of the essential oil of cinnamon is cinnamaldehyde. The content of cinnamaldehyde (86.5%) identified for cinnamon essential oil in our results is similar to LI et al., (2013) (66.2-81.9), higher than LV et al., (2013) (77.3%) and lower than ZHANG et al., (2016) (92.4%). Our results highlighted the significant higher activity ($P < 0.05$) of cinnamon essential oil when compared to ceftiofur for *S. aureus* and *T. pyogenes*. *T. pyogenes* is considered one of the most important pathogens causing endometritis in dairy cows and cephalosporin-based drugs are most commonly used as treatments in cows

with endometritis (LEBLANC, 2008, SHELDON and OWENS, 2017). In addition, it is important to emphasize that cinnamon essential oil showed significantly higher antibacterial activity ($P < 0.05$) than the other essential oils (clove, eucalyptus, lemon, oregano, rosemary and thyme) investigated in the present study against the pathogenic species of *F. necrophorum* and *T. pyogenes*. Studies regarding antibacterial activity of essential oils in relation to these two pathogenic species causing endometritis have not been found. This is the first study to show the *in vitro* activity of essential oils in potentially endometritis-causing bacteria. According to our results, it was demonstrated the promising potential of cinnamon essential oil as natural therapy in cows with endometritis.

Based on the results of the clove essential oil components, our results are in accordance with literature data, which show that eugenol (> 85%) is the major component identified (CHAIEB et al., 2007; BHUIYAN et al., 2010). 1,8-cineol (> 80%) was the main component identified in eucalyptus essential oil, corroborating the results of previous study (SACCHETTI et al., 2005). According to the identification of the lemon essential oil components, the main constituents identified were limonene (65.5%) and β-pinene (15%), which is in agreement with the study by HSOUNA et al., (2017) (39.7% and 25.44, respectively). Carvacrol (>70%) was the major component of oregano essential oil, similar results were described by EBANI et al., (2017) (65.90%) and FRATINI et al., (2017) (65.94%). The main constituent of the rosemary essential oil was 1,8-cineol (47%) and is similar to that described by YANG et al., (2010) (46%). The main compound identified in thyme essential in oil in this study was thymol (48%), our results are in agreement with those described by SOKOVIC et al., (2009) (48%) and EBANI et al., (2017) (52%). Different growing environments such as altitude, hours of sunshine, temperature, rainfall, and parts of the plant extracted for the supply of essential oil may contribute to the difference between the percentages of identified active components (WANG et al., 2009; HOSSAIN et al., 2014).

The results of this study shown that the essential oils tested have different activity against the bacteria evaluated considering the IZD observed. To date, there have been no reports in the literature on the use of essential oils against strains of *T. pyogenes* and *F. necrophorum* strains.

Several authors have reported the antibacterial activity of *Cinnamon cassia* essential oil (NIMJE et al., 2013; MELO et al., 2015; CIESLAK et al., 2016; ZHANG et al., 2016; ZHU et al., 2016). Our results of IZD of cinnamon essential oil against *E. coli* (32 mm) are similar to

those described by NIMJE et al., (2013) (32 mm), MELO et al., (2015) (30 mm) and ZHU et al., (2016) (30 mm), and larger than those described by ZHANG et al., (2016) (19 mm). Based on the results observed in the present study the IZD (38 mm) of cinnamon essential oil against *S. aureus*, our results are in agreement with those described by MELO et al., (2015) (40 mm) and CIESLAK et al., (2016) (35 mm), and larger than those described by ZHU et al., (2016) (29 mm), ZHANG et al., (2016) (28 mm) and NIMJE et al., (2013) (21 mm). The main component of *Cinnamon cassia* oil used in this study was cinnamaldehyde (86%). The antibacterial activity of *Cinnamon cassia* essential oil is mainly due to the cinnamaldehyde component, that have hydrophobic properties, and can react with bacterial cell membranes, contributing to damage the membrane, another action is the ability to inhibit bacterial peptide and protein synthesis, thus having gram-positive and gram-negative bacteria action (ZHU et al., 2016; RUTALA and WEBER, 2008).

The antibacterial effects of clove essential oil have been described in the literature (PERINI et al., 2014). Our results of IZD of clove essential oil against *E. coli* (9 mm) were smaller than those noted by OULKHEIR et al., (2017) (16 mm), PRABUSEENIVASAN et al., (2006) (17 mm) and BARTKIENE et al., (2018) (11 mm). The IZD of clove essential oil against *S. aureus* (15 mm) noted in this study were similar than those described by PRABUSEENIVASAN et al., (2006) (16 mm) and BARTKIENE et al., (2018) (16 mm). The main component of clove essential oil was eugenol (85%), this compound is responsible for the antibacterial effect of clove essential oil. The eugenol has the ability to denature proteins and react with cell membrane phospholipids, altering membrane permeability (PERINI et al., 2014).

Our study showed least inhibitory activity of eucalyptus essential against *E. coli* (8 mm) and *S. aureus* (8 mm). FRATINI et al., (2017) also did not observe IZD results using eucalyptus essential oil against *S. aureus* and *E. coli*.

HSOUNA et al., (2017) using lemon essential oil noted IZD against the reference strain of *E. coli* (15 mm) and *S. aureus* (22 mm). However, in the present study no antibacterial activity was identified against the bacteria tested.

Previous studies showed the antibacterial activity of oregano essential oil (MARQUES et al., 2015; FRATINI et al., 2017). Our results of IZD of essential oil of oregano against *E. coli* (20 mm) were smaller than those noted by MELO et al., (2015) (38 mm), while against *S. aureus*, our results of IZD (36 mm) are larger than those described by EBANI et al., (2017) (13 mm). The major constituent of oregano essential oil in this study was carvacrol (72%). The

main mechanism of action of carvacrol against the bacterial cell is the collapse of the proton motor force, the depletion of the ATP pool, and may act on the phospholipid bilayer of the cell membrane, increasing the permeability and leakage of vital intracellular components, which can cause membrane disruption and contribute to cell death (EBANI et al., 2017).

The antibacterial activity of rosemary essential oil has been previously reported (JIANG et al., 2011). Our results of IZD of rosemary oil against *E. coli* (8 mm) and *S. aureus* (10 mm) were smaller than those showed by PRABUSEENIVASAN et al., (2006) (17 mm and 12 mm, respectively). The differences might be related with distinct composition of the essential oils tested.

The high antimicrobial activity of thyme essential oil has been previously revealed (SZWEDA et al., 2018). Thyme essential oil showed a range of IZD of 24–36 mm in this study. These results are in agreement with those reported by Oulkheir et al., (2017) that showed activity of thyme essential oil against *E. coli* (18 mm) and *S. aureus* (22 mm). Thymol (48%), the main compound of thyme essential oil, have been found to exhibit antimicrobial activity (COSTA et al., 2013), acting on the membrane of bacteria, contributing to the release of lipopolysaccharides, increasing the permeability of the cell membrane, and increasing the loss of ATP and the leakage of vital intracellular constituents (EBANI et al., 2017).

7.6 CONCLUSION

This study revealed that essential oils have antibacterial activity against the main bacteria tested causing endometritis. Therefore, essential oils have great potential as an alternative to be explored as endometritis therapy in dairy cows. Further *in vivo* studies are recommended to evaluate the use in clinical applications.

7.7 REFERENCES

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6 GENERAL CONCLUSIONS

The results of this Thesis evidenced the main microorganisms present in the uterine environment of dairy cows with and without uterine diseases during the postpartum period. There was a significantly higher isolation rate of *Trueperella pyogenes* in CE cows compared to healthy and SE cows. Furthermore, our results demonstrated that dairy cows affected by clinical endometritis and subclinical endometritis had worse reproductive performance and milk production than healthy cows. Finally, we highlight a promising antibacterial activity of essential oils of cinnamon, thyme, oregano and clove against the main bacteria associated with uterine diseases in dairy cows.

The results of this Thesis provide important information about uterine diseases in Brazilian herds, which may contribute to a greater understanding of local veterinarians and technicians due to the lack of information about these diseases in Brazil, promoting the rational and selective use of antibiotics in dairy herds.

SUPPLEMENTARY TABLES

Table S1: Distribution of bacterial isolation profiles for cows with SE (subclinical endometritis) and CE (clinical endometritis).

Group	Isolation Profile		N	%
SE	*Aerococcus viridans*		3	14.29
	Aerococcus viridans	*Paenibacillus cookii*	1	4.76
	Aerococcus viridans	*Streptococcus pluranimalium*	1	4.76
	Bacillus cereus		3	14.29
	Bacillus cereus	*Clostridium perfringens*	1	4.76
	Bacillus cereus	*Streptococcus uberis*	1	4.76
	Escherichia coli	*Pseudomonas stutzeri*	1	4.76
	Escherichia coli	*Pseudomonas fulva*	1	4.76
	Pseudomonas fulva		1	4.76
	Pseudomonas rhodesiae		1	4.76
	Corynebacterium efficiens		1	4.76
	Micrococcus luteus		1	4.76
	Paenibacillus cookii		1	4.76
	Streptococcus pluranimalium		1	4.76
	Staphylococcus warneri		1	4.76

	Staphylococcus chromogenes	*Paenibacillus phoenicis*	*Scedosporium apiospermum*	*Bacillus licheniformis*	*Bacillus siralis*	1	4.76
	Stenotrophomonas maltophilia	*Acinetobacter radioresistens*	*Bacillus megaterium*			1	4.76
	Total					21	100.0
CE	*Aerococcus viridans*					3	4.41
	Aerococcus viridans	*Histophilus somni*				1	1.47
	Aerococcus viridans	*Actinomyces hyovaginalis*				1	1.47
	Aerococcus viridans	*Bacillus licheniformis*				1	1.47
	Aerococcus viridans	*Staphylococcus xylosus*				1	1.47
	Aerococcus viridans	*Staphylococcus epidermidis*	*Lysinibacillus xylanilyticus*			1	1.47
	Aerococcus viridans	*Enterococcus hirae*	*Staphylococcus xylosus*	*Bifidobacterium pseudolongom*		1	1.47
	Bacillus cereus					6	8.82
	Bacillus cereus	*Escherichia coli*				1	1.47
	Bacillus cereus	*Paenibacillus residui*				1	1.47
	Bacillus cereus	*Pseudomonas fulva*				1	1.47
	Bacillus cereus	*Aerococcus viridans*	*Streptococcus pneumoniae*	*Streptococcus sanguinis*		1	1.47
	Escherichia coli					3	4.41

	Escherichia coli	*Staphylococcus epidermidis*			1	1.47
	Escherichia coli	*Aerococcus viridans*			1	1.47
	Escherichia coli	*Enterococcus gallinarum*			1	1.47
	Escherichia coli	*Clostridium perfringens*	*Pseudomonas fulva*		1	1.47
	Escherichia coli	*Klebsiella oxytoca*	*Pseudomonas monteilii*	*Enterobacter cloacae*	1	1.47
	Escherichia coli	*Klebsiella oxytoca*	*Pseudomonas stutzeri*	*Streptococcus pluranimalium*	1	1.47
	Trueperella pyogenes				4	5.88
	Trueperella pyogenes	*Escherichia coli*			3	4.41
	Trueperella pyogenes	*Helcococcus ovis*			2	2.94
	Trueperella pyogenes	*Helcococcus kunzii*			1	1.47
	Trueperella pyogenes	*Prevotella heparinolytica*			2	2.94
	Trueperella pyogenes	*Bacteroides pyogenes*			1	1.47
	Trueperella pyogenes	*Helcococcus ovis*	*Fusobacterium necrophorum*		1	1.47
	Trueperella pyogenes	*Enterococcus villorum*	*Lysinibacillus xylanilyticus*		1	1.47
	Trueperella pyogenes	*Porphyromonas levii*	*Prevotella heparinolytica*	*Dermabacter hominis*	1	1.47
	Trueperella pyogenes	*Bacillus cereus*	*Escherichia coli*	*Streptococcus salivarius*	1	1.47

	Trueperella pyogenes	*Enterococcus aerogenes*	*Enterobacter cloacae*	*Siccibacter turicensis*		1	1.47
	Trueperella pyogenes	*Escherichia coli*	*Porphyromonas levii*	*Staphylococcus epidermidis*		1	1.47
	Trueperella pyogenes	*Escherichia coli*	*Helcococcus ovis*	*Fusobacterium necrophorum*	*Klebsiella pneumoniae*	1	1.47
	Trueperella pyogenes	*Prevotella heparinolytica*	*Stenotrophomonas maltophilia*	*Bifidobacterium pseudolongom*	*Delftia acidovorans*	1	1.47
	Trueperella pyogenes	*Porphyromonas levii*	*Bacteroides pyogenes*	*Staphylococcus warneri*	*Enterococcus villorum*	1	1.47
	Staphylococcus xylosus					3	4.41
	Staphylococcus arlettae					1	1.47
	Staphylococcus hominis	*Micrococcus luteus*				1	1.47
	Staphylococcus warneri	*Rothia amarae*				1	1.47
	Staphylococcus xylosus	*Paenibacillus cookii*				1	1.47
	Staphylococcus xylosus	*Corynebacterium xerosis*				1	1.47
	Pseudomonas aeruginosa					1	1.47
	Pseudomonas fulva	*Bacillus licheniformis*				1	1.47
	Pseudomonas rhodesiae	*Acinetobacter beijerinakii*				1	1.47
	Bacteroides pyogenes	*Paenibacillus turicensis*				1	1.47

	Clostridium diolis			1	1.47
	Lactobacillus agilis			1	1.47
	Micrococcus luteus			1	1.47
	Streptococcus sanguinis			1	1.47
	Stenotrophomonas maltophilia	*Paenibacillus macerans*		1	1.47
	Enterococcus faecium	*Paenibacillus cookii*	*Lysinibacillus massiliensis*	1	1.47
	Total			68	100.0

Table S2: Distribution of bacterial isolation profiles among studied healthy cows.

Group	Isolation Profile					N	%
Healthy	*Aerococcus viridans*					3	2.88
	Aerococcus viridans	*Staphylococcus xylosus*				3	2.88
	Aerococcus viridans	*Staphylococcus sciuri*	*Leclercia adecarboxylata*			1	0.96
	Aerococcus viridans	*Stenotrophomonas maltophilia*	*Actinomyces odontolyticus*			1	0.96
	Aerococcus viridans	*Stenotrophomonas maltophilia*	*Corynebacterium stationis*			1	0.96
	Aerococcus viridans	*Staphylococcus hominis*	*Paenibacillus amynolyticus*	*Siccibacter turicensis*		1	0.96
	Aerococcus viridans	*Streptococcus dysgalactiae*	*Enterococcus casseliflavus*	*Staphylococcus xylosus*		1	0.96
	Aerococcus viridans	*Vagococcus fluvialis*	*Staphylococcus sciuri*	*Proteus hauseri*		1	0.96
	Aerococcus viridans	*Escherichia coli*	*Enterococcus faecium*	*Enterococcus mundtii*	*Enterococcus saccarolyticus*	1	0.96
	Bacillus cereus					5	4.81

	Bacillus cereus	*Lysinibacillus fusiformis*	*Brevibacillus laterosporus*		1	0.96
	Bacillus cereus	*Aerococcus viridans*	*Staphylococcus arlettae*	*Corynebacterium xerosis*	1	0.96
	Bacillus coagulans	*Micrococcus lylae*			1	0.96
	Bacillus licheniformis				2	1.92
	Bacillus licheniformis	*Bacillus oleronius*			1	0.96
	Bacillus thuringiensis				1	0.96
	Escherichia coli				4	3.85
	Escherichia coli	*Staphylococcus chromogenes*			1	0.96
	Escherichia coli	*Enterobacter cloacae*			1	0.96
	Escherichia coli	*Enterobacter xiangfangensis*			1	0.96
	Escherichia coli	*Kocuria marina*			1	0.96
	Escherichia coli	*Bacillus licheniformis*			1	0.96
	Escherichia coli	*Klebsiella variicola*			1	0.96

	Escherichia coli	*Paenibacillus barcinonensis*					1	0.96
	Escherichia coli	*Klebsiella oxytoca*	*Acinetobacter schindleri*				1	0.96
	Escherichia coli	*Staphylococcus xylosus*	*Staphylococcus sciuri*				1	0.96
	Escherichia coli	*Streptococcus lutetiensis*	*Staphylococcus chromogenes*	*Streptococcus alactolyticus*			1	0.96
	Escherichia coli	*Enterococcus gallinarum*	*Staphylococcus arlettae*	*Enterococcus italicus*	*Enterococcus thailandicus*	*Bifidobacterium pseudolongom*	1	0.96
	Escherichia coli	*Enterococcus faecium*	*Enterococcus hirae*	*Citrobacter koseri*	*Citrobacter amalonaticus*	*Micrococcus luteus*	1	0.96
	Escherichia coli	*Pseudomonas monteilii*	*Enterobacter cloacae*	*Escherichia hermannii*	*Leclercia adecarboxylata*	*Bacillus pumilus*	1	0.96
	Pseudomonas fulva	*Escherichia coli*	*Clostridium perfringens*				3	2.88
	Pseudomonas putida	*Klebsiella oxytoca*	*Enterobacter cloacae*				1	0.96
	Pseudomonas stutzeri						1	0.96

	Pseudomonas stutzeri	*Salmonella enterica*				1	0.96
	Staphylococcus arlettae	*Corynebacterium flavencens*				1	0.96
	Staphylococcus arlettae	*Streptococcus lutetiensis*	*Staphylococcus warneri*			1	0.96
	Staphylococcus arlettae	*Staphylococcus xylosus*	*Enterococcus avium*	*Bacillus licheniformis*		1	0.96
	Staphylococcus chromogenes	*Corynebacterium stationis*				1	0.96
	Staphylococcus epidermidis					1	0.96
	Staphylococcus haemolyticus					1	0.96
	Staphylococcus xylosus					4	3.85
	Staphylococcus xylosus	*Staphylococcus sciuri*	*Aerococcus viridans*	*Enterococcus saccarolyticus*	*Brachybacterium conglomeratum*	1	0.96
	Stenotrophomonas maltophilia					5	4.81

	Stenotrophomonas maltophilia	*Enterococcus faecium*				1	0.96
	Stenotrophomonas maltophilia	*Bacillus altitudinis*	*Microbacterium paraoxydans*	*Bifidobacterium pseudolongom*		1	0.96
	Streptococcus alactolyticus	*Streptococcus lutetiensis*				1	0.96
	Streptococcus dysgalactiae	*Atopobium minutum*				1	0.96
	Streptococcus pluranimalium					3	2.88
	Streptococcus pluranimalium	*Stenotrophomonas maltophilia*				1	0.96
	Streptococcus pluranimalium	*Escherichia coli*	*Staphylococcus caprae*	*Staphylococcus arlettae*	*Streptococcus alactolyticus*	1	0.96
	Trueperella pyogenes	*Streptococcus mitis*				1	0.96
	Brachybacterium conglomeratum	*Staphylococcus sciuri*				1	0.96
	Brevibacillus agri					1	0.96
	Brevibacillus parabrevis	*Paenibacillus phoenicis*				1	0.96

	Citrobacter freundii					1	0.96
	Citrobacter freundii	*Stenotrophomonas maltophilia*	*Delftia acidovorans*			1	0.96
	Corynebacterium efficiens					1	0.96
	Corynebacterium glutamicum					1	0.96
	Corynebacterium xerosis	*Bifidobacterium pseudolongom*				1	0.96
	Corynebacterium xerosis	*Aerococcus viridans*	*Cellulosimicrobium cellulans*			1	0.96
	Corynebacterium xerosis	*Corynebacterium jeikeium*	*Enterobacter kobei*	*Brevibacterium luteolum*	*Staphylococcus xylosus*	1	0.96
	Cutibacterium acnes					1	0.96
	Cutibacterium acnes	*Brevibacillus parabrevis*				1	0.96
	Enterobacter cloacae					1	0.96

	Enterobacter cloacae	*Leclercia adecarboxylata*				1	0.96
	Enterococcus durans					1	0.96
	Enterococcus faecalis					1	0.96
	Enterococcus faecium	*Enterococcus casseliflavus*				1	0.96
	Enterococcus faecium	*Escherichia coli*	*Lysinibacillus massiliensis*	*Paenibacillus barcinonensis*	*Bacillus oleronius*	1	0.96
	Enterococcus gallinarum	*Pantoea ananatis*	*Paenibacillus amynolyticus*			1	0.96
	Klebsiella oxytoca					1	0.96
	Kocuria rhizophila	*Pantoea agglomerans*				1	0.96
	Lysinibacillus fusiforms					1	0.96
	Macrococcus canis	*Roseomonas mucosa*				1	0.96
	Microbacterium oxydans	*Microbacterium esteraromaticum*				1	0.96

	Micrococcus luteus			1	0.96
	Paenibacillus cookii			1	0.96
	Paenibacillus cookii	*Brevibacillus parabrevis*		1	0.96
	Paenibacillus illinoisensis	*Bacillus licheniformis*	*Bacillus clausii*	1	0.96
	Paenibacillus macerans			1	0.96
	Pluralibacter gergoviae			1	0.96
	Total			104	100.0

APPENDIX

APPENDIX A

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Original Article

Chemical composition and antibacterial activity of essential oils against pathogens often related to cattle endometritis

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Abstract

Introduction: Endometritis is a condition marked by inflammation of the endometrium that affects dairy cows from 21 days after parturition, causing damage to herd fertility and economic losses on farms. The use of active compounds obtained from plant sources has gained importance as disease treatment agents in farm animals due to the high resistance rates currently observed against traditional antibiotics commonly used. The study was carried out to examine the chemical composition and to investigate the antibacterial activity of rosemary, cinnamon, cloves, eucalyptus, lemon, oregano and thyme essential oils against the reference strain of *Escherichia coli* (ATCC 25922), *Fusobacterium necrophorum* (ATCC 25286), *Trueperella pyogenes* (ATCC 19411) and *Staphylococcus aureus* (ATCC 29213), considered as typical bacteria causing endometritis.

Methodology: The chemical composition of the seven essential oils were analyzed by GC-MS and their antibacterial activity was evaluated by the disc diffusion method.

Results: Thirty-six components were identified in total using GC-MS analyzes. The main compounds were cinnamaldehyde (86.5% for cinnamon essential oil), eugenol (85.7% for clove essential oil), 1,8-cineol (80% for eucalyptus and 47.8% rosemary essential oils), limonene (65.5% for lemon essential oil), carvacrol (72.1% for oregano essential oil) and thymol (48.8% for thyme essential oil). The disc diffusion assay revealed that cinnamon, clove, oregano, and thyme essential oils showed the best results compared to the other three essential oils, showing the largest zone of inhibition against all bacteria evaluated.

Conclusions: These findings indicated that essential oils are a potential agent to be used as an alternative for bovine endometritis treatment.

Key words: Bovine endometritis; essential oils; antimicrobial activity; phytotherapy.

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