UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CAMPUS DE BOTUCATU

DINÂMICA DA PREVALÊNCIA DE PATÓGENOS CAUSADORES DE MASTITE EM
PROPRIEDADES LEITEIRAS BRASILEIRAS DE 2012 A 2020 E ASSOCIAÇÃO COM
ANO E ESTAÇÃO DO ANO

LUCIANA SEKITO DE FREITAS ZAMBELLI

Dissertação apresentada ao Programa de
Pós-graduação em Zootecnia como parte das
exigências para obtenção do título de Mestre
em Zootecnia

BOTUCATU – SP
Setembro de 2023

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**BIOGRAFIA DO AUTOR**

Luciana Sekito de Freitas Zambelli nascida em 15 de julho de 1979 no município de São Paulo, estado de São Paulo, filha de Libia Harumi Sekito de Freitas e Jordão de Oliveira Freitas, casada com Carlos Alexandre Zambelli desde 2008 e mãe da Sabrina Sekito Zambelli nascida em 2012 e Rebeca Sekito Zambelli nascida em 2015. Aos 11 anos, mudou-se com a família do Brasil para Lisboa, Portugal onde estudou até 1996. No Brasil, finalizou seus estudos do ensino médio e, em 1998, mudou-se para Botucatu para cursar Medicina Veterinária na Faculdade de Medicina Veterinária e Zootecnia – FMVZ, da Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP. De 1998 a 2002, atuou intensamente em diversas atividades acadêmicas, sendo membro da empresa júnior de consultoria agropecuária Conapec Jr. atuando como Diretora Administrativa, participante e monitora de fazenda de gado leiteiro da região de Botucatu. Atuou também como Diretora Administrativa do Diretório Acadêmico da Medicina Veterinária Walter Maurício Corrêa, organizadora da XV Semana de Estudos Agropecuários e Florestais de Botucatu em 2001 do módulo "Empresa leiteira: Aspectos fundamentais de sanidade e produção animal", e participou de plantão clínico voluntário no Hospital Veterinário da FMVZ e campanha de vacinação antirrábica. Em 2000 e 2001, envolveu-se em pesquisa e desenvolvimento no Departamento de Moléstias Infecciosas (MI) da FMVZ desenvolvendo um projeto de Iniciação Científica em imunologia em búfalas sob orientação do Prof. Márcio Garcia Ribeiro. Em 2020, realizou estágio curricular obrigatório na University of Wisconsin (UW), nos EUA, no laboratório de Qualidade do Leite do Departamento de Dairy Sciences em projetos relacionados à qualidade do leite, mastite subclínica e características dos testes de diagnóstico, sob supervisão da Dra. Pamela L. Ruegg. Na sua carreira corporativa em multinacionais, trabalhou na Gerência de Produto e Assuntos Regulatórios na área de saúde e nutrição animal, coordenação e suporte a projetos regionais e globais de inovação para a área de biotecnologia na agricultura aplicada ao controle biológico de nematóides, tornando-se um dos inventores de patentes na área de nematicidas biológicos em diversos países como EUA, Alemanha, Brasil, México e países na Ásia. Em 2021, após anos dedicando-se ao mundo corporativo, à maternidade e à consultoria autônoma de Assuntos Regulatórios, ingressou no Mestrado do Programa de Pós-graduação em Zootecnia da FMVZ, UNESP, campus de Botucatu para desenvolver pesquisa em agentes causadores de mastite em propriedades leiteiras brasileiras sob orientação do Prof. Dr. José Eduardo Portela Santos da University of Florida (UF). Em 2022, foi visitante escolar no Department of Animal Sciences da UF em Gainesville, FL, EUA

para análise estatística dos dados do projeto, escrita de artigo científico e colaboração nas atividades do laboratório do seu orientador.

## DEDICATÓRIA

Dedico esta dissertação aos amores da minha vida Carlos, Sabrina e Rebeca e a todos que contribuem intensamente para a produção de leite de qualidade no Brasil.

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Aos meus pais que são exemplos de dedicação, persistência e capricho em tudo que se propõem a fazer e por sempre acreditarem em mim; a meu pai pelo encorajamento de retomar a carreira profissional e suas habilidades de planejamento e execução, e a minha mãe, pelo devotamento à família e sua admirável disciplina.

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Muitíssimo obrigada!

"Pode ser difícil superar o vento, o frio e a falta de oxigênio, mas é muito mais difícil superar-se a si mesmo."

(Waldemar Niclevicz)

**RESUMO GERAL**

Os objetivos foram determinar o perfil e a prevalência de patógenos contagiosos e ambientais causadores de mastite em amostras de leite de vacas com mastite clínica ou subclínica, ou de programas de monitoramento sanitário do úbere submetidas a um laboratório de diagnóstico comercial ao longo de 9 anos. Este estudo longitudinal observacional analisou o perfil microbiológico de 717.168 resultados microbiológicos de 3.793 fazendas leiteiras de 2012 a 2020. Análises subsequentes foram realizadas em um subconjunto de dados de 204.461 resultados microbiológicos de 12 fazendas que submeteram amostras em todos os 9 anos. Amostras de leite originaram-se de quartos individuais ou amostras compostas. Um resultado de crescimento foi definido quando 3 ou mais colônias de 1 a 3 microrganismos diferentes estavam presentes no meio de cultura, exceto para *Staphylococcus aureus* e *Streptococcus agalactiae* em que uma colônia foi considerada crescimento. Um resultado contaminado foi definido quando uma ou mais colônias de 4 ou mais microorganismos diferentes estavam presentes no meio de cultura. Nas amostras das 3.793 fazendas, a prevalência de patógenos causadores de mastite contagiosa foi de 28,7% em 2012 e 26,6% em 2020, com o verão tendo a maior prevalência, 27,6%. A prevalência de patógenos ambientais causadores de mastite foi de aproximadamente 26,0% em 2012 e 2020, sendo o outono e a primavera os de maior prevalência, 27,5%. A prevalência de *Staphylococcus aureus* não diferiu entre 2012 e 2020, 10,9% e 10,3%, respectivamente, e a estação do ano esteve associada à prevalência. A prevalência de *Streptococcus agalactiae* diminuiu de 7,19% em 2012 para 3,54% em 2020, e o inverno teve a maior prevalência, 7,73%, em comparação com outono, primavera ou verão. Os patógenos causadores de mastite mais prevalentes foram estafilococos não-aureus (SNA), *Staphylococcus chromogenes*, *Staphylococcus aureus,* *Streptococcus agalactiae,* *Corynebacterium bovis*, *Klebsiella* spp, *Streptococcus uberis*, *Escherichia coli*, *Streptococcus dysgalactiae* e *Pseudomonas* spp. As 12 fazendas que submeteram amostras de leite em todos os 9 anos mostraram uma diminuição na prevalência de patógenos causadores de mastite contagiosa de 15,1% em 2012 para 8,02% em 2020. De fato, a prevalência de *S. agalactiae* diminuiu de 5,66% em 2013 para 0,21% em 2020. Similarmente, a prevalência de patógenos ambientais causadores de mastite diminuiu de 38,2% em 2012 para 25,1% em 2020, com o verão apresentando a maior prevalência, 34,5%. No Brasil, foi observada uma pequena redução gradual na prevalência de patógenos contagiosos causadores de mastite, mas não foi observada uma mudança no perfil de patógenos contagiosos para patógenos ambientais nas 3.793 fazendas. Por outro lado, o subconjunto de fazendas comprometidas com a identificação de

patógenos causadores de mastite reduziu a prevalência de *S. agalactiae* e mostrou uma mudança no perfil de patógenos contagiosos em relação aos patógenos causadores de mastite ambientais. Produtores comprometidos com a identificação de patógenos causadores de mastite provavelmente implementarão medidas específicas de controle para reduzir a prevalência de bactérias contagiosas que causam mastite.

**Palavras-chave**:contagiosa, ambiental, mastite, microrganismos, prevalência

**ABSTRACT**

Objectives were to determine the profile and prevalence of contagious and environmental mastitis-causing pathogens in milk samples from cows experiencing clinical or subclinical mastitis, or from udder health monitoring programs submitted to a commercial diagnostic laboratory over a period of 9 years. This observational longitudinal study analyzed the microbiological profile from 717,168 milk samples from 3,793 dairy farms from 2012 to 2020. Further analyses were performed in a subset of 204,461 microbiological results of 12 farms that submitted samples in all 9 years. Milk samples originated from individual quarters or composite samples. A growth result was defined when 3 or more colonies from 1 to 3 different pathogens were present in culture media, except for *Staphylococcus aureus* and *Streptococcus agalactiae* which 1 colony was defined as growth. A contaminated result was defined when 1 or more colonies from 4 or more microorganisms were present in culture media. In samples from the 3,793 farms, the prevalence of contagious mastitis-causing pathogens was 28.7% in 2012 and 26.6% in 2020, with summer having the greatest prevalence, 27.6%. The prevalence of environmental mastitis-causing pathogens was approximately 26.0% in 2012 and 2020, and fall and spring seasons had the greatest prevalence, 27.5%. The prevalence of *S. aureus* did not differ between 2012 and 2020, 10.9% and 10.3%, respectively, but season was associated with the prevalence. The prevalence of *S. agalactiae* decreased from 7.19% in 2012 to 3.54% in 2020, and winter had the greatest prevalence, 7.73%, compared with fall, spring, or summer. The most prevalent mastitis-causing pathogens were non-*aureus* staphylococci (NAS), *Staphylococcus chromogenes*, *S. aureus*, *S. agalactiae*, *Corynebacterium bovis*, *Klebsiella* spp, *Streptococcus uberis*, *Escherichia coli*, *Streptococcus dysgalactiae*, and *Pseudomonas* spp. The 12 farms that submitted milk samples in all 9 years had a reduction in the prevalence of contagious mastitis-causing pathogens from 15.1% in 2012 to 8.02% in 2020. Indeed, the prevalence of *S. agalactiae* decreased from 5.66% in 2013 to 0.21% in 2020. Similarly, the prevalence of environmental mastitis-causing pathogens in milk samples decreased from 38.2% in 2012 to 25.1% in 2020, with summer presenting the greatest prevalence, 34.5%. A small gradual reduction in the prevalence of contagious mastitis-causing pathogens was observed in dairy farms in Brazil, but a change in the profile from contagious to environmental pathogens was not detected in the 3,793 farms. Conversely, the subset of farms committed to the identification of mastitis-causing pathogens had a reduction in the prevalence of *S. agalactiae* and experienced a change in the profile towards environmental mastitis-causing pathogens.

Producers committed to the identification of mastitis-causing pathogens are likely to implement specific control measures to reduce the prevalence of contagious bacteria that cause mastitis.

**Key words**: contagious, environmental, mastitis, microorganisms, prevalence

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**CAPÍTULO 2**

## LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

| | |
|---|---|
| ANVISA | Agência Nacional de Vigilância Sanitária |
| AOR | "Adjusted odds ratio" |
| BTM | "Bulk tank milk" |
| cel | Célula |
| CMT | "California Mastitis Test" |
| CAMP | Christie, Atkins, Munch-Petersen |
| CAPES | Coordenação de Aperfeiçoamento de Pessoal de Nível Superior |
| CCS | Contagem de células somáticas |
| CI | "Confidence interval" |
| CM | "Clinical mastitis" |
| d | Dia |
| FMVZ | Faculdade de Medicina Veterinária e Zootecnia |
| EUA | Estados Unidos da América |
| FL | Flórida |
| IA | Inseminação artificial |
| kg | Quilograma |
| l | "Liter" |
| L | Litro |
| LSM | "Least-squares means" |
| MC | Mastite clínica |
| MI | Moléstias infecciosas |
| ml | "Milliliter" |
| mL | Mililitro |
| MAPA | Ministério da Agricultura, Pecuária e Abastecimento |
| MSC | Mastite subclínica |
| NAHMS | National Animal Health Monitoring System |
| NAS | "Non-aureus staphylococci" |
| NMC | National Mastitis Council |
| OR | "Odds ratio" |
| SCC | "Somatic cell count" |
| SCM | "Subclinical mastitis" |

| | |
|---|---|
| SEM | "Standard errors of the means" |
| SNA | Staphylococci não-aureus |
| STROBE | "The Strengthening the Reporting of Observational Studies in Epidemiology" |
| UF | University of Florida |
| UNESP | Universidade Estadual Paulista Júlio de Mesquita Filho |
| UW | University of Wisconsin |

# SUMÁRIO

# CAPÍTULO 1

**Considerações iniciais**

A mastite continua sendo uma das principais doenças em rebanhos leiteiros (NAHMS, 2021) associada a impactos negativos na produção de leite, reprodução e sobrevivência de vacas leiteiras (SCHRICK *et al.*, 2001; SANTOS *et al.*, 2004), gerando grande perda econômica para a indústria de laticínio, incluindo custos de tratamento e descarte de leite e vacas, aumento na mão-de-obra e diminuição da qualidade e composição do leite (BLOSSER, 1979; DOHOO e MEEK, 1982; DEGRAVES e FETROW, 1993). A perda econômica associada à mastite foi estimada, nos Estados Unidos da América (EUA), em 2 bilhões de dólares anuais (DEGRAVES e FETROW, 1993), e na Austrália, em 110 milhões de dólares por ano e em torno de 260 dólares por caso de mastite (DYSON *et al.*, 2022).

A mastite tem diversidade etiológica, frequentemente, associada a patógenos (WATTS, 1988) dos quais alguns são altamente prevalentes com características microbiológicas específicas (SMITH *et al.*, 1985; FOX e GAY, 1993). Conhecer o agente causador da mastite permite a implementação de medidas de controle específicas relacionadas à sua terapia e prevenção (RUEGG, 2003 e DOHOO *et al.*, 2011).

A mastite permanecerá relevante em futuras pesquisas mesmo com os avanços, desenvolvimento e implantação de programas de controle do último século, devido às mudanças na estrutura dos rebanhos e exigências de rigorosos padrões de qualidade e processos (RUEGG, 2017).

Países ou rebanhos no mundo que, consistentemente, adotaram estratégias de prevenção e terapia específica controlaram ou erradicaram os patógenos contagiosos causadores de mastite como *Staphylococcus aureus*, *Streptococcus agalactiae* e *Corynebacterium bovis* dos rebanhos leiteiros, como observado nos EUA, em alguns países da Europa, Nova Zelândia e Austrália (MAKOVEC and RUEGG, 2003; KOIVULA *et al.*, 2007; PIEPERS *et al.*, 2007; DYSON *et al.*, 2022). Rebanhos que dão ênfase a programas de controle de mastite costumam observar uma mudança na prevalência de agentes bacterianos da doença, com redução na proporção do isolamento de patógenos contagiosos e aumento na proporção de patógenos ambientais (DEGRAVES and FETROW, 1993; RUEGG, 2017).

No Brasil, estudos regionais estimaram a prevalência de *S. aureus* variando de 4,0% a 19,2%, *S. agalactiae* de 7,0% a 16,9% e *Corynebacteria* de 9,0% a 55,2% (BRITO *et al.*, 1999; LANGONI *et al.*, 2011; BARBOSA *et al.*, 2017; TOMAZI *et al.*, 2018, DALANEZI *et al.*, 2019). Um estudo observacional com 517 rebanhos leiteiros que submeteram amostras de leite para contagem de células somáticas (CCS) entre os anos de 2011 e 2015, com 8.285 dias-teste das 5 regiões do Brasil, relatou uma prevalência de mastite subclínica, ou seja, amostras de leite com ≥ 200.000 células/mL, de 51,2% em 2015 (BUSANELLO *et al.*, 2017). Sabe-se que

rebanhos com CCS de tanque de leite de média a alta, ou seja, entre 151.000 e 400.000 células/mL apresentam maior proporção de mastite subclínica causada por patógenos contagiosos do que rebanhos com menos de 150.000 células/mL (BARKEMA *et al.*, 1998). Esses resultados sugerem que a mastite contagiosa não está erradicada ou controlada em rebanhos leiteiros de determinadas regiões do país.

Estudos epidemiológicos observacionais permitem uma avaliação inicial qualificável para diagnosticar a situação e implementar programas de controle e prevenção de patógenos causadores de mastite. Embora, a habilidade de realizar inferências gerais causais sobre uma população seja limitante, principalmente quando os estudos divergem nos resultados (RUEGG, 2017; DOLECHECK *et al.*, 2019).

O presente estudo longitudinal abrange diversas áreas de produção de leite no Brasil, incluindo amostras das 5 regiões geográficas do país durante as 4 estações do ano, proporcionando assim a oportunidade de caracterizar a etiologia bacteriana da mastite bovina em rebanhos leiteiros brasileiros que representa o situação do rebanho leiteiro no país no período estudado.

A hipótese deste estudo é que a prevalência de patógenos de mastite contagiosa e ambiental em rebanhos leiteiros brasileiros permanece relevante e que a mudança do perfil de mastite contagiosa para ambiental ainda não ocorreu como observada em alguns países. Este estudo longitudinal teve como objetivo determinar, durante um período de 9 anos, a dinâmica da prevalência e o perfil de patógenos causadores de mastite isolados de amostras de leite de vacas com mastite ou de programas de monitoramento da saúde do úbere submetidos a um laboratório de diagnóstico comercial e sua associação com ano e estação de ano.

## 1   Revisão de Literatura

### 1.1 Definição e importância econômica da mastite

A mastite é definida como a inflamação do tecido da glândula mamária, geralmente causada por infecção por microrganismos presentes no ambiente em que a vaca vive ou na glândula mamária de animais infectados, tais como bactérias, fungos, leveduras e algas. De 137 microrganismos isolados da glândula mamária, no mínimo 84 podem causar mastite em vacas leiteiras (WATTS, 1988). Estes patógenos invadem, primariamente, a glândula mamária pelo canal do teto, se reproduzem e estimulam uma resposta inflamatória (SCHRICK *et al.*, 2001).

Devido à sua diversidade etiológica e alta prevalência nos rebanhos leiteiros, a mastite tem sido considerada uma das doenças mais relevantes na produção de leite associada a impactos

negativos na produtividade de leite, saúde, bem-estar e sobrevivência dos animais (SANTOS *et al.*, 2004).

Segundo um estudo do United States Department of Agriculture (USDA) com um grupo de 265 fazendas leiteiras com mais de 30 animais em lactação em cada rebanho e provenientes dos 17 estados mais relevantes para a produção de leite, a mastite foi considerada uma das mais importantes doenças em vacas leiteiras. O estudo reportou um aumento de casos de mastite clínica entre 1996 e 2014 de 13,0% para 24,8%, respectivamente, e um aumento na taxa de tratamento de 15,0% dos casos de mastite em 2002 para 22,0% dos casos em 2014 (NAHMS, 2021).

As perdas econômicas causadas pela mastite incluem custos de tratamento, descarte do leite e de animais, aumento na mão-de-obra e diminuição da qualidade e composição do leite, além da redução na produção de leite (BLOSSER, 1979; DOHOO e MEEK, 1982; SANTOS *et al.*, 2004). Estas perdas foram estimadas em 2 bilhões de dólares anuais nos EUA (DEGRAVES e FETROW, 1993) e na Austrália, em torno de 110 milhões de dólares anuais e 260 dólares em casos individuais de mastite (DYSON *et al.*, 2022).

Um estudo conduzido por Aghamohammadi *et al.* (2018) com 145 produtores de leite candanenses e considerando diferentes componentes de custos associados à mastite reportou um custo de 66.178 dólares canadenses por 100 vacas por ano e 662 dólares canadenses por vaca em lactação. A redução na produção de leite foi o componente que mais contribuiu para as perdas econômicas, seguido pelo descarte e implementação de medidas preventivas. A mastite subclínica foi responsável por 48% do custo total estimado, sendo que deste, 72% foi atribuído à redução da produção de leite, 25% atribuído ao descarte de animais, e o restante atribuído ao leite descartado, serviços veterinários e diagnóstico da mastite. Em contraste, a mastite clínica contribuiu com 34% do custo total, distribuído em 48% dos custos relativos ao descarte e mortalidade, 34% devido à redução da produção de leite, e o restante distribuído em descarte do leite e mão-de-obra (AGHAMOHAMMADI *et al.,* 2018).

Outros trabalhos (BLOSSER, 1979; DEGRAVES e FETROW, 1993) também observaram que a redução na produção do leite é o maior componente do prejuízo da mastite. Esta perda de produção varia com o patógeno causador e o número de casos de mastite (HERTL *et al.*, 2014).

1.2 Mastite e eficiência reprodutiva

Dolecheck *et al.* (2019) realizaram uma revisão sistemática da literatura seguida de meta-análise incluindo 29 estudos observacionais publicados entre 1998 e 2017 que avaliaram as associações entre mastite de ocorrência espontânea e desempenho reprodutivo de vacas leiteiras. Os autores concluíram que há uma associação quantificável entre incidência de mastite e a

eficiência reprodutiva de vacas leiteiras e que a prevenção da mastite é importante para melhorar o desempenho produtivo e reprodutivo das vacas e a rentabilidade dos rebanhos leiteiros.

Em um estudo de desempenho reprodutivo com 1.001 vacas pós-parto provenientes de duas fazendas comercias e com 320 dias em lactação, SANTOS *et al*. (2004) reportaram que vacas que apresentaram mastite clínica antes da primeira inseminação artificial (IA) pós-parto tiveram uma redução de 2,2 kg/d de leite, menor porcentagem de prenhez na primeira IA, menor taxa de prenhez, maior incidência de aborto e maior número de dias em aberto em comparação a vacas que não desenvolveram mastite. Vacas diagnosticadas com mastite entre a primeira IA pós-parto e o diagnóstico de prenhez tiveram redução de 1,4 kg/d de leite na lactação (SANTOS *et al.*, 2004).

Em um outro estudo de desempenho reprodutivo com 752 vacas Jersey, com um grupo controle de vacas sem mastite clínica ou subclínica, um grupo de vacas com mastite clínica (MC) e outro grupo com mastite subclínica (MSC) antes da primeira IA, e um grupo de vacas que desenvolveu mastite clínica após um caso de mastite subclínica, foi observado um aumento em torno de 20 dias para primeira IA pós-parto, de 25 dias em aberto e de 0,5 no número de serviços/prenhez no grupo de vacas com mastite em comparação ao grupo controle (SCHRICK *et al*., 2001).

No Brasil, um estudo realizado por Dalanezi *et al*. (2019) com 833 vacas leiteiras provenientes de 5 rebanhos comerciais demonstrou que vacas acometidas por mastite clínica apresentaram mais dias em aberto comparadas ao grupo controle que não teve caso de mastite; vacas com mastite causada por patógenos maiores, como *S. aureus, S. agalactiae, E. coli, Klebsiella* spp*, Mycoplasma* spp e estreptococos ambientais, precisaram de um número maior de IA comparadas às do grupo de patógenos menores como staphylococci não-aureus (SNA) e *Corynebacterium* spp. A perda de prenhez no grupo controle foi menor do que no grupo de patógenos maiores (DALANEZI *et al*., 2019).

1.3 Mastite clínica e subclínica

A mastite pode ser classificada como clínica, em que os sinais de alteração no leite e úbere são visíveis como inchaço, edema, presença de grumos ou pus no leite, ou até sinais sistêmicos na vaca, e dependendo da gravidade destes sinais a mastite é classificada como leve, moderada e grave, ou pode ser subaguda, aguda, hiperaguda ou crônica dependendo do aparecimento e duração da mastite (BARKEMA *et al*., 1998; TOMAZI *et al.,* 2018).

A mastite clínica é diagnosticada pela observação dos sinais acima descritos e é mais comum em vacas multíparas do que primíparas, podendo apresentar casos recorrentes na mesma lactação ou entre várias lactações (HERTL *et al*., 2014).

Rebanhos com programas bem estabelecidos de controle de patógenos contagiosos, com CCS de leite de tanque abaixo de 220.000 células/mL e que mantém boas práticas de higiene e ambiência tendem a apresentar desafios com a mastite clínica causada por patógenos Gram-negativos como *Escherichia coli*, *Klebsiella* spp e *Pseudomonas* spp (GONZALES *et al.*, 1990; BARKEMA *et al.*, 1998). A mastite clínica também é causada por patógenos Gram-positivos, como demonstrou um estudo de 65 rebanhos da região sudeste da Austrália (DYSON *et al.*, 2022) durante um período de 14 meses com 2.572 amostras de leite de vacas com mastite clínica, em que os autores relataram isolamento mais comum de *Streptococcus uberis* (39,2%), *S. aureus* (10,6%) e *S. dysgalactiae* (6,4%). No Brasil, um estudo realizado por Tomazi *et al.* (2018) com 20 rebanhos leiteiros da região Sudeste durante um período de 8 a 15 meses reportou que, de 4.212 amostras de leite coletadas de vacas com mastite clínica, os patógenos mais frequentemente isolados foram a *E. coli* (6,6%), o *S. uberis* (6,1%) e o *S. agalactiae* (5,9%).

A mastite subclínica é caracterizada pela ausência de alteração visível no leite e no úbere de vacas e um dos métodos utilizados para seu diagnóstico e monitoramento é a CCS do leite individual e do leite do tanque de refrigeração que apresentam mais de 200.000 células/mL de leite (DOHOO e LESLIE, 1991).

As estimativas de prevalência e incidência da mastite subclínica são essencias para determinar a dimensão do problema e avaliar a eficácia das medidas de controle implantadas em um rebanho leiteiro (RUEGG, 2003). As medidas de controle específicas implantadas e o monitoramento constante dos índices de mastite subclínica reduziram a média geométrica da CCS do leite do tanque de refrigeração, na Bélgica, de mais de 300.000 células/mL em 1991 para 196.000 células/mL em 1999; porém, desde 2000, houve um aumento na média geométrica da CCS do leite do tanque de refrigeração para 221.000 células/mL em 2006 (PIEPERS *et al.*, 2007). Os autores atribuíram este crescimento à presença de *S. aureus* em 25% dos 1.087 rebanhos leiteiros estudados, e aos SNA que foram os pátogenos mais frequentemente isolados em 41,1% dos rebanhos com CCS $\geq$ 250.000 células/mL.

Um estudo com amostras de leite de tanque de refrigeração de 894 fazendas leiteiras analisadas de 3 províncias chinesas realizado por Bi *et al.* (2016) reportou isolamento de *S. agalactiae, S. dysgalactiae e S. aureus,* em 824 (92,2%), 647 (72,3%) e em 448 (50,1%) amostras de leite e uma alta correlação destes resultados com a média geométrica dos tanques de refrigeração de 650.000 células/mL. Entre os principais patógenos ambientais, o estudo reportou isolamento de *Trueperella pyogenes* em 601 (67,2%), *Enterococcus* spp em 319 (35,7%), *E. coli* em 255 (28,6%) e *Klebsiella* spp em 178 (20,0%) amostras de leite de tanque analisadas e não houve relação significativa entre a CCS de leite de tanque e os patógenos ambientais, exceto para *T. pyogenes* (BI *et al.*, 2016).

No Brasil, um estudo epidemiológico com 8.285 dias de teste de 517 rebanhos das cinco regiões do país, considerando a prevalência de mastite subclínica como o número de vacas com CCS ≥ 200.000 células/mL dividido pelo número total de vacas testadas em um determinado dia de teste, foi estimada em 51,2% (BUSANELLO *et al.*, 2017). Langoni *et al.* (2011) reportaram grandes diferenças na ocorrência de mastite subclínica de 1,44% a 47,8% ao avaliar dez propriedades do estado de São Paulo e 1090 tetos com isolamento predominante de *C. bovis* (29,5%), *Streptococcus dysgalactiae* (11,9%) e *S. aureus* (10,5%).

### 1.4 Mastite contagiosa

A classificação da mastite também pode ser feita considerando o reservatório em que os microrganismos causadores de mastite habitam. Os patógenos contagiosos têm, primariamente, a glândula mamária ou tecidos adjacentes como reservatório e são transmitidos entre as vacas durante a ordenha quando os tetos são expostos a bactérias presentes no leite ordenhado de quartos infectados com estes patógenos que incluem o *Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium bovis* e *Mycoplasma* spp (FOX e GAY, 1993).

As infecções por patógenos contagiosos apresentam características gerais como fácil cultivo em placas de ágar sangue, exceto os micoplasmas que exigem microaerofilia e meios de cultivo específicos segundo o National Mastitis Council (ADKIN *et al.*, 2017); não são resultado de contaminação durante a coleta de amostra de leite de vacas com mastite ou suspeita; quando não tratadas, desenvolvem casos de mastite de longa duração predominantemente subclínicas e seu monitoramento deve incluir cultivo de leite de vacas com mastite clínica, cultivo de leite de tanque de refrigeração, e avaliação da contagem de células somáticas de leite de tanque ou amostras compostas individuais das vacas mensalmente (SMITH *et al.*, 1985; BARKEMA *et al.*, 1998).

As práticas de controle da mastite contagiosa são definidas para prevenir novas infecções e eliminar as existentes (RUEGG, 2003) e incluem uso de solução de pré-imersão e pós-imersão de tetos, também conhecidas como pré- e pós-*dipping*, terapia da vaca seca, manutenção do equipamento de ordenha, tratamento correto dos casos clínicos e subclínicos, descarte das vacas crônicas, e implementação de programas de qualidade do leite para obter sucesso no controle ou erradicação dos patógenos contagiosos (RODRIGUES e RUEGG, 2005).

### 1.4.1 *Staphylococcus aureus*

O *S. aureus* é uma bactéria Gram-positiva, beta-hemolítica, catalase e coagulase positivas (NMC, 1990) e está presente na maioria dos rebanhos leiteiros no mundo, apesar das medidas de controle e prevenção direcionadas aos contagiosos (ØSTERÅ *et al.*, 2006, FERGUSON *et al.*,

2007; LANGONI *et al.*, 2011 e GAO *et al.*, 2016;). O *S. aureus* possui características específicas impondo diversos fatores predisponentes à infecção e cronicidade, como resistência a muitos antibióticos beta-lactâmicos por produzirem a enzima beta-lactamase, colonização da pele do teto, aderência às células epiteliais por uma ligação específica às proteínas da matriz extracelular fibronectina e colágeno, formação de microabscessos e biofilmes, além da presença de outros fatores de virulência como Proteína A, cápsulas e toxinas conferindo proteção aos fatores bactericidas endógenos e exógenos (FOX e GAY, 1993). Este patógeno também possui mecanismos de evasão do sistema imune do hospedeiro como neutralização da opsonização, da fagocitose e da morte citotóxica por neutrófilos através da ação da leucocidina citotóxica LukMF' (CÔTE-GRAVEL e MALOUIN, 2019).

Na China, Bi *et al.*, (2016) reportaram isolamento do *S. aureus* em 50,1% de 894 amostras de leite de tanque de refrigeração de propriedades leiteiras de 3 regiões chinesas. Em um estudo regional com 1087 rebanhos e 178.668 amostras de leite de quartos, na Bélgica, o *S. aureus* foi o patógeno maior mais isolado de quartos subclinicamente infectados com 25% de prevalência (PIEPERS *et al.*, 2007). Na Itália, *S. aureus* foi isolado de 20,6% de 18.711 amostras de leite de quartos coletados de vacas leiteiras com mastite clínica ou subclínica da região da Sicília durante 6 anos (FERGUSON *et al.*, 2007), enquanto na Alemanha, TENHAGEN *et al.* (2006) reportaram prevalência de 5,7% de um total de 10.034 amostras de leite de 96 rebanhos de uma base de dados de conveniência. Nos EUA, em um estudo observacional, também de uma base de dados de conveniência de 77.172 amostras de leite submetidas a um laboratório de diagnóstico em Wisconsin durante 6 anos, a prevalência foi de 9,7% em 2001 (MAKOVEC e RUEGG, 2003).

No Brasil, estudos estimaram a prevalência de *S. aureus* entre 1,3% e 19,2% (BRITO *et al.*, 1999; LANGONI *et al.*, 2011; BARBOSA *et al.*, 2017; TOMAZI *et al.*, 2018, DALANEZI *et al.*, 2019). Esta variação pode ser atribuída a diferenças regionais, tamanho da amostragem, aos critérios de coleta de amostras de leite com base no tipo de mastite, clínica ou subclínica; originárias de quarto ou coleta composta, ou da escolha do método de diagnóstico de mastite subclínica, como California Mastitis Test (CMT) ou CCS.

1.4.2 *Streptococcus agalactiae*

O *S. agalactiae* é uma bactéria Gram-positiva, beta-hemolítica, catalase e esculina negativas e CAMP (Christie, Atkins, Munch-Petersen) positiva (ADKIN *et al.*, 2017).

Historicamente, nos EUA (MAKOVEC e RUEGG, 2003) e Europa (KOIVULA *et al.*, 2007; PIEPERS *et al.*, 2007), as ações de controle e tratamento iniciais adotadas nas fazendas leiteiras foram direcionadas aos patógenos contagiosos de forma consistente, principalmente para *S. agalactiae* cuja erradicação é possível e deve ser atingida nos rebanhos leiteiros, implantando a

*blitz* terapia que consiste em identificar e tratar os quartos infectados das vacas acometidas (DODD *et al*., 1969).

Na Finlândia, a prevalência da mastite subclínica decresceu de 48% (1988) para 31% (2001) e a erradicação de *S. agalactiae* foi observada com prevalência de 0,1% em 77.051 amostras de leite coletadas de janeiro de 2004 a janeiro de 2006 (KOIVULA *et al*., 2007).

A erradicação de *S. agalactiae* também foi observada na Bélgica em 178.668 amostras de leite de quartos de vacas analisadas durante 3 anos (PIEPERS *et al*., 2007). Makovec e Ruegg (2003) reportaram resultados similares em 77.172 amostras de leite recebidas em um laboratório em Wisconsin, EUA entre os anos de 1994 e 2001 em que a prevalência de *S. agalactiae* decresceu de 8,1% (1994) para 3,0% (2001). Os autores também observaram que patógenos ambientais e contagiosos demonstraram diferenças características entre as estações do ano em que *Streptococcus agalactiae* apresentou maior probabilidade de isolamento no inverno ao longo dos anos comparado com as outras estações. Entretanto, *E. coli* e *Klebsiella* spp apresentaram menor probabilidade de isolamento no inverno comparado às demais estações do ano (MAKOVEC e RUEGG, 2003).

Na Alemanha, Tenhagen *et al*. (2006) realizaram um estudo com 9.910 amostras de leite coletadas de 2.529 vacas, entre julho de 2001 a outubro de 2002, e reportaram a prevalência de *S. agalactiae* em 2,7%. Porém, é importante mencionar que este patógeno foi isolado em 29% de 80 propriedades leiteiras participantes do estudo (TENHAGEN *et al.*, 2006).

No Brasil, estudos regionais estimaram a prevalência de *S. agalactiae* entre 4,1% e 16,9% (BRITO *et al*., 1999; LANGONI *et al*., 2011; BARBOSA *et al*., 2017; TOMAZI *et al*., 2018, DALANEZI *et al*., 2019).

Relatos recentes documentaram uma reemergência do *S. agalactiae* em fazendas em países nórdicos, como a Dinamarca que, no início dos anos 1960 apresentava prevalência de aproximadamente 40% em nível de rebanho; em 1989, após implementação de programas de monitoramento e controle deste patógenos, apresentou 1%, mas em 2012, ocorreu um aumento da prevalência para 5% (CHURAKOV *et al*., 2021). Crestani *et al*. (2021) sugeriram uma relação desta reemergência com as vias de transmissão de humanos para animais, como peixes e bovinos. Jørgensen *et al*. (2016) não consideram o *S. agalactiae* um patógeno obrigatório da glândula mamária, pois este pode ser encontrado no trato gastrointestinal e consideram uma possível transmissão oro-fecal via ambiente e água.

### 1.4.3 *Corynebacterium bovis*

*Corynebacterium bovis* é uma bactéria Gram-positiva, não-hemolítica, catalase positiva considerada moderadamente patogênica, associada a respostas inflamatórias moderadas (ADKIN *et al*., 2017). Dentre o grupo das *Corynebacterium* spp, *C. bovis* é a mais

frequentemente isolada de amostras de leite de vacas (GONÇALVES *et al*., 2016; LÜCKEN, *et al*., 2022). Esta bactéria pode ser facilmente controlada dos rebanhos através de práticas de higiene durante a ordenha como pós-imersão e terapia da vaca seca (FOX e GAY, 1993).

*Corynebacterium bovis* tem sido descrita como patógeno menor ou comensal, colonizador, principalmente do canal do teto e cisterna do teto; os quartos infectados com esta bactéria podem ser mais resistentes a infecções por *S. aureus* do que quartos negativos e mais susceptíveis a *S. agalactiae* do que quartos negativos (PANKEY *et al*., 1984).

Gonçalves *et al*. (2016) realizaram um estudo observacional no Brasil com 21 rebanhos leiteiros e 285 amostras de leite de quartos previamente diagnosticados com *Corynebacterium* spp para determinar associação da mastite subclínica por *C. bovis* e a qualidade do leite produzido, comparando quartos contralaterais saudáveis e infectados com esta bactéria. Os autores relataram que quartos infectados por *C. bovis* apresentaram um leve aumento na CCS de 87.770 células/mL para 174.280 células/mL quando comparados aos quartos contralaterais saudáveis ou infectados por outros agentes bacterianos, apesar destes valores estarem abaixo do valor de corte de 200.000 células/mL para indicar um processo inflamatório na glândula mamária; também observaram a diminuição no conteúdo de lactose e de sólidos do leite não-gordurosos de vacas infectadas, porém nenhuma associação foi observada com a produção de leite, gordura, proteína e sólidos totais (GONÇALVES *et al*., 2016).

Em diversos países do mundo, *C. bovis* está controlada e não tem sido isolada de amostras de leite com frequência (MAKOVEC e RUEGG, 2003; ØSTERÅS *et al*., 2006; KOIVULA *et al*., 2007; DYSON *et al*., 2021), porém Tenhagen *et al*. (2006) reportou isolamento de *C. bovis* em 7,3% em amostras de leite de vacas de 80 rebanhos na Alemanha, e Gao *et al*. (2017) reportaram prevalência de 12,4% de 3.288 amostras de leite de mastite clínica de 161 propriedades leiteiras com mais de 500 vacas leiteiras na China.

No Brasil, estudos regionais estimaram a prevalência de *C. bovis* entre 9,0% e 55,2% (BRITO *et al*., 1999; LANGONI *et al*., 2011; BARBOSA *et al*., 2017; TOMAZI *et al*., 2018).

1.5 Mastite ambiental

Ao contrário da mastite contagiosa, a mastite ambiental é causada por uma diversidade de microrganismos cuja fonte primária é o ambiente em que as vacas vivem e a transmissão ocorre, primariamente, entre as ordenhas (SMITH *et al*., 1985). Provavelmente, a contaminação do teto entre ordenhas aumenta o risco de entrada destes microrganismos na cisterna do teto e eventualmente no tecido mamário durante o processo de ordenha. Os principais patógenos neste grupo são os bacilos entéricos Gram-negativos como *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* e *Enterococcus* spp e os estreptococos ambientais dentre eles, *Streptococcus uberis* (WATTS, 1988). Os patógenos ambientais estão presentes nos rebanhos leiteiros, pois

não são bem controlados pelas medidas adotadas para contagiosos como pós-imersão, descarte de vacas crônicas e higiene do equipamento de ordenha, e sim medidas como pré-imersão e manutenção de um ambiente limpo e seco para os animais (ROSSITTO *et al*., 2002).

Uma mudança ao longo do tempo do perfil da mastite contagiosa para ambiental em rebanhos bem manejados tem sido observada (DEGRAVES E FETROW, 1993; RUEGG, 2017).

Um estudo realizado na Finlândia, com 77.051 amostras de leite coletadas em um banco de dados nacional divididos em 14.190 amostras de leite de mastite clínica e 7.762 de mastite subclínica, reportou prevalência de *S. uberis de* 14,6% para casos de mastite clínica e 11,6% para casos de mastite subclínica; *E. coli* foi isolada de 5,95% dos casos clínicos e 1,64% dos casos subclínicos (KOIVULA *et al*., 2007); Makovec e Ruegg (2003) observaram prevalências de 20,1% de estreptococos ambientais e 6,7% de *E. coli* dentre 77.172 amostras de leite de vacas com mastite submetidas ao laboratório de diagnóstico em Wisconsin, EUA. Um estudo recente realizado na Austrália, os autores reportaram uma prevalência de 39,2% para *S. uberis* e 8,4% para *E. coli* em 2.752 amostras de leite de casos clínicos de mastite (DYSON *et al*., 2022), sugerindo que, historicamente, o controle e a prevenção destes patógenos são mais complexos e difíceis quando comparados aos contagiosos (SMITH *et al*., 1985).

No Brasil, Barbosa *et al*. (2017) reportaram que *E. coli* e *Klebsiella* spp foram os patógenos ambientais mais isolados dentre 1.397 amostras compostas de leite de vacas com prevalências de 11,7% e 8,8%, respectivamente. No mesmo ano, um estudo com 20 rebanhos leiteiros no estado de São Paulo e 4.212 amostras de leite de quartos de vacas com mastite clínica, reportou prevalência de 6,6% para *E. coli* e 6,1% para *S. uberis* (TOMAZI *et al*., 2018). Em um outro estudo, *S. dysgalactiae* foi o patógeno ambiental mais isolado dentre 1.090 amostras de leite de quartos de 10 fazendas leiteiras com mastite clínica ou subclínica, com prevalência de 11,9% (LANGONI *et al.*, 2011).

1.6 Staphylococci não-aureus

Os SNA são bactérias Gram-positivas, catalase positivas, coagulase negativa ou positiva dependendo da espécie, como o *Staphylococcus hyicus* e o *Staphylococcus agnetis* que por anos foram incluídos no grupo denominado estafilococos coagulase negativa (ADKIN *et al*., 2017; DE BUCK *et al*., 2021).

Os SNA são um grupo vasto com diferentes espécies de estafilococos e o mais frequentemente isolado de infecções intramamárias em vacas leiteiras, como reportado em diversos estudos em diferentes partes do mundo, sendo o *Staphylococcus chromogenes* o mais isolado do grupo (FERGUSON *et al*., 2007; BARBOSA *et al*., 2017, GAO *et al*., 2017). Koivula *et al*. (2007) reportaram prevalências de 21,1% (n = 77.051), 17,6% (n = 14.190) e 23,5% (n =

7.762) de SNA em amostras de leite totais, amostras de casos clínicos e subclínicos, respectivamente.

Em um estudo na Bélgica com 13 rebanhos leiteiros e 624 amostras de leite de quartos, *S. chromogenes, Staphylococcus sciuri* e *Staphylococcus cohnii* foram reportados como as espécies mais predominantes nas infecções intramamárias, e o *S. chromogenes* foi encontrado em todos os rebanhos estudados e o único associado a mastite em novilhas recém-paridas e vacas em lactação (DE VISSCHER *et al*., 2016).

No Canadá, em um estudo coorte, com 5.507 isolados de SNA coletados de 3.561 quartos com CCS < 200.00 células/mL, 1.873 quartos com CCS >200.000 células/mL e 73 casos de mastite clínica (CONDAS *et al*., 2017), foi reportado que os SNA mais prevalentes, como *S. chromogenes, Staphylococcus simulans, Staphylococcus xylosus, Staphylococcus haemoliyticus, Staphylococcus epidermidis,* estavam associados a quartos com CCS > 200.000 células/mL. A prevalência reportada de SNA foi 18,5% em casos de mastite subclínica sendo que *S. agnetis, Staphylococcus capitis, S. hyiucus, Staphylococcus gallinarum* e *S. simulans* aumentaram mais a CCS, apesar de ser um aumento pequeno, e prevalência de 4,3% em casos de mastite clínica com isolamento mais comum de *S. chromogenes, S. simulans* e *S. sciuri* (CONDAS *et al*., 2017).

SNA é um grupo diversificado e estudos longitudinais em larga escala podem ser úteis para entender como as diferenças de cepas e sua evolução afetam a prevalência e a distribuição de SNA causadores de mastite em vacas leiterias, o impacto causado na saúde da glândula mamária e as associações entre as medidas de controle de mastite específicas para este grupo (DE BUCK *et al*., 2021).

1.7 Método laboratorial para diagnóstico de mastite

O conhecimento da distribuição do patógeno causador da infecção mamária, tanto a clínica como a subclínica, nos rebanhos leiteiros é essencial para definir protocolos de tratamento e identificar as práticas de manejo mais adequadas para reduzir novas infecções e eliminar as existentes (DOHOO *et al*., 2011; DYSON *et al*., 2022).

A cultura microbiológica em placa convencional associada a testes bioquímicos em laboratórios credenciados, historicamente, é considerada padrão-ouro para diagnóstico microbiológico do agente causador de mastite e amplamente utilizada em pesquisas científicas no mundo todo (HARMON *et al*., 1990; RUEGG, 2017).

Estudos epidemiológicos observacionais permitem uma avaliação inicial qualificável para diagnosticar a situação e implementar programas de controle e prevenção de patógenos causadores de mastite. Embora, a habilidade de realizar inferências gerais causais sobre uma população seja limitante, principalmente quando os estudos divergem nos resultados (RUEGG,

2017; DOLECHECK *et al*., 2019). Entretanto, eles permitem entender a situação atual do problema e, dependendo do desenho do estudo, permite identificar os fatores de risco para os agentes causadores de mastite e as possíveis medidas de prevenção e controle.

O presente trabalho elaborado nesta dissertação é um estudo longitudinal que abrange as mais diversas áreas produtoras de leite do Brasil, incluindo amostras das 5 regiões geográficas do país durante as 4 estações do ano permitindo assim identificar a etiologia bacteriana da mastite bovina em rebanhos brasileiros com boa validade externa. Portanto, os resultados presentes nesta dissertação permitem fazer inferências sobre a situação da mastite bovina contagiosa e da ambiental no país.

O presente estudo longitudinal abrange diversas áreas de produção de leite no Brasil, incluindo amostras das 5 regiões geográficas do país durante as 4 estações do ano, proporcionando assim a oportunidade de caracterizar a etiologia bacteriana da mastite bovina em rebanhos leiteiros brasileiros que representa o situação do rebanho leiteiro no país no período estudado.

A hipótese do meu trabalho é que a prevalência de patógenos de mastite contagiosa e ambiental em rebanhos leiteiros brasileiros permanece relevante e que a mudança do perfil de mastite contagiosa para ambiental ainda não ocorreu como observado em alguns países.

Este estudo longitudinal teve como objetivo determinar, durante um período de 9 anos, a dinâmica da prevalência e o perfil de patógenos causadores de mastite isolados de amostras de leite de vacas com mastite ou de programas de monitoramento da saúde do úbere submetidos a um laboratório de diagnóstico comercial e sua associação com ano e estação do ano.

O artigo científico deste estudo será submetido à revista científica Journal of Dairy Science.

**Referências**

ADKINS, P. R. F. *et al.* **Laboratory Handbook on Bovine Mastitis**. New Prague, MN: National Mastitis Council, Inc. 2017.

AGHAMOHAMMADI, M. *et al.* Herd-level mastitis associated costs on Canadian dairy farms. **Front. Vet. Sci.**, Lausanne, v. 5, n. 100, p.1-12, 2018. DOI 10.3389/fvets.2018.00100. Disponível em https://www.frontiersin.org/articles/10.3389/fvets.2018.00100/full. Acesso em: 07 mai. 2023.

BARBOSA, L. F. S. P. *et al.* Somatic cell count and type of intramammary infection impacts fertility from *in vitro* produced embryo transfer. **Theriogenology**, Amsterdam, v. 108, p. 291-296, 2017. DOI 10.1016/j.theriogenology.2017.12.025. Disponível em: https://pubmed.ncbi.nlm.nih.gov/29277069. Acesso em: 11 jun. 2021.

BARKEMA, H. W. *et al.* Incidence of clinical mastitis in dairy herds grouped in three categories by bulk milk somatic cell counts. **J. Dairy Sci.,** Champaign, v. 81, n. 2, p. 411-419, 1998. DOI 10.3168/jds.S0022-0302(98)75591-2. Disponível em: https://pubmed.ncbi.nlm.nih.gov/9532494. Acesso em: 13 out. 2020.

BI Y. *et al.* Prevalence of Bovine Mastitis Pathogens in Bulk Tank Milk in China. **PLoS ONE** v. 11, n. 5, p. 1-13, 2016. DOI 10.1371/journal.pone.0155621. Disponível em: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0155621. Acesso em: 10 mai. 2023.

BLOSSER, T. H. Economic losses from and the national research program on mastitis in the United States. **J. Dairy Sci.,** Champaign, v. 62, n. 1, p. 119-127, 1979. DOI 10.3168/jds.S0022-0302(79)83213-0. Disponível em: https://pubmed.ncbi.nlm.nih.gov/379057. Acesso em: 06 dez. 2022.

BRITO, M. A. V. P. *et al.* Dairy herds pattern of intramammary infection: evaluation of all mammary quarters of lactating cows. **Arq. Bras. Med. Vet. Zootec.**, Belo Horizonte, v. 51, n. 2, p. 129-135, 1999. DOI 10.159/S01102-09351999000200001. Disponível em: https://www.scielo.br/j/abm vz/a/9Fpg49DMSf9RKNjp8fSf54x/?lang=pt. Acesso em: 10 abr. 2023.

BUSANELLO, M. *et al.* Estimation of prevalence and incidence of subclinical mastitis in a large population of Brazilian dairy herds. **J. Dairy Sci.**, Champaign, v. 100, n. 8, p. 6545–6553, 2017.

DOI https://doi.org/10.3168/jds.2016-12042. Disponível em: https://www.journalof dairyscience.org/article/S0022-0302(17)30582-9. Acesso em: 13 out. 2020.

CHURAKOV *et al*. Assessing potential routes of *Streptococcus agalactiae* transmission between dairy herds using national surveillance, animal movement data and molecular typing. **Prev. Vet. Med.,** Amsterdam, v. 197, p. 105501, 2021. DOI 10.1016/j.prevetmed .2021.105501. Disponível em: https://pubmed.ncbi.nlm.nih.gov/34624567. Acesso em: 09 mai. 2023.

CONDAS *et al*. Distribution of non-*aureus* staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. **J. Dairy Sci.**, Champaign, v. 100, n. 7, p. 1-15, 2017. DOI 10.3168/jds.2016-12479. Disponível em: https://pubmed.ncbi.nlm.nih.gov /28456402. Acesso em: 12 jan. 2023.

CÔTE-GRAVEL, J.; MALOUIN, F. Symposium review: Features of *Staphylococcus aureus* mastitis pathogenesis that guide vaccine development strategies. **J. Dairy Sci.**, Champaign, v. 102, n. 5, p. 4727-4740, 2019. DOI 10.3168/jds.2018-15272. Disponível em: https://www. sciencedirect.com/science/article/pii/S0022030218311007#bib68. Acesso em: 03 ago. 2023.

CRESTANI C. *et al*. The fall and rise of group b *Streptococcus* in dairy cattle: reintroduction due to human-to-cattle host jumps. **Microb. Genom.**, London, v. 7, n. 000648, p. 1-10, 2021. DOI 10.1099/mgen.0000648. Disponível em: https://pubmed.ncbi.nlm.nih.gov/34486971. Acesso em: 10 mai. 2023.

DALANEZI, F. M. *et al*. Influence of pathogens causing clinical mastitis on reproductive variables of dairy cows. **J. Dairy Sci.**, Champaign, v. 103, n. 4, p. 3648-3655, 2019. DOI 10.3168/jds.2019-16841. Disponível em: https://pubmed.ncbi.nlm.nih.gov/32089296. Acesso em: 21 abr. 2022.

DE BUCK *et al*. Non-*aureus Staphylococci* and bovine udder health: current understanding and knowledge gaps. **Front. Vet. Sci.**, Amsterdam, v. 8, art. 658031, p. 1-16, 2021. DOI 10.3389/fvets.2021.658031. Disponível em: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC 8081856. Acesso em: 26 mai. 2022.

DEGRAVES, F. J.; FETROW, J. Economics of mastitis and mastitis control. **Vet. Clin. North Am. Food Anim. Pract.**, Philadelphia, v. 9, n. 3, p. 421–434, 1993. DOI 10.1016/s0749-0720(15)30611-3.

DE VISSCHER *et al*. Intramammary infection with coagulase-negative *staphylococci* at parturition: species-specific prevalence, risk factors, and effect on udder health. **J. Dairy Sci.**, Champaign, v. 99, n. 8, p. 6457-6469, 2016. DOI 10.3168/jds.2015-10458. Disponível em: https://pubmed.ncbi.nlm.nih.gov/27236763. Acesso em: 24 abr. 2023.

DODD, F. H. *et al*. Mastitis – the strategy control. **J. Dairy Sci.**, Champaign, v. 52, n. 5, p. 689-695, 1969. DOI 10.3168/jds.S0022-0302(69)86631-2. Disponível em: https://pubmed. ncbi.nlm.nih.gov/5391702. Acesso em: 07 dez. 2022.

DOHOO, I. R.; LESLIE K. E. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. **Prev. Vet. Med.**, Amsterdam, v. 10, p. 225-237, 1991. DOI. 10.1016/0167-5877(91)90006-N. Disponível em: https://www.sciencedirect.com/science/ article/pii/016758779190006N. Acesso em: 09 mai. 2023.

DOHOO, I. R.; MEEK, A. H. Somatic cell counts in bovine milk. **Can. Vet. J.,** Ottawa, v. 23, n. 4, p. 119-125, 1982. Disponível em: https://pubmed.ncbi.nlm.nih.gov/17422127/. Acesso em: 06 dez. 2022.

DOHOO, I. R. *et al*. Diagnosing intramammary infections: evaluation of definitions based on a single milk sample. **J. Dairy Sci.**, Champaign, v. 94, n. 1, p. 250-261, 2011. DOI 10.3168/jds.2010-3559. Disponível em: https://pubmed.ncbi.nlm.nih.gov/21183035. Acesso em: 02 mar. 2021.

DOLECHECK, K. A. *et al*. Quantifying the effects of mastitis on the reproductive performance of dairy cows: A meta-analysis. **J. Dairy Sci.**, Champaign, v. 102, n. 9, p. 8454-8477, 2019. DOI 10.3168/jds.2018-15127. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S00 2203021930534X. Acesso em: 11 jun. 2021.

DYSON, R. *et al*. A survey of mastitis pathogens including antimicrobial susceptibility in southeastern Australian dairy herds. **J. Dairy Sci.**, Champaign, v. 105, n. 2, p. 1504-1518, 2022.

DOI 10.3168/jds.2021-20955. Disponível em: https://pubmed.ncbi.nlm.nih.gov /34955276. Acesso em: 29 nov. 2022.

FERGUSON, J. D., *et al*. Prevalence of mastitis pathogens in Ragusa, Sicily, from 2000 to 2006. **J. Dairy Sci.**, Champaign, v 90, n. 12, p. 5798-5813, 2007. DOI 10.3168 /jds.2006-903. Disponível em: https://www.sciencedirect.com/science/article/pii/S0022030207720568. Acesso em: 03 mai, 2023.

FOX, L. K.; GAY, J. M. Contagious mastitis. **Vet. Clin. North Am. Food Anim. Pract.**, Philadelphia, v. 9, n. 3, p. 475-487, 1993. DOI 10.1016/s0749-0720(15)30615-0. Disponível em: https://pubmed.ncbinlm.nih.gov/8242453. Acesso em: 05 dez. 2022.

GAO J., *et al*. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. **J. Dairy Sci.**, v. 100, n. 6, p. 4797-4806, 2017. DOI 10.3168/jds.2016-12334. Disponível em: https://www.journalofdairyscience.org/article/S0022-0302(17)30333-8/pdf. Acesso em: 29 nov. 2022.

GONÇALVES, J. L. *et al*. Effects of bovine subclinical mastitis caused by *Corynebacterium* spp on somatic cell count, milk yield and composition by comparing contralateral quarters. **Vet J**, London, v. 209, p. 87-92, 2016. DOI 10.1016/j.tvjl.2015.08.009. Disponível em: https://pubmed.ncbi.nlm.nih.gov/26831159. Acesso em: 11 mai. 2023.

GONZALEZ, R. N. *et al*. Clinical mastitis in two California dairy herds participating in contagious mastitis control programs. **J. Dairy Sci.,** Champaign, v. 73, n. 3, p. 648-660, 1990. DOI 10.3168/jds.S0022-0302(90)78716-4. Disponível em: https://pubmed.ncbi.nlm.nih.gov /2341640. Acesso em: 05 dez. 2022.

HARMON, R. J. *et al*. 1990. **Microbiological Procedures for the diagnosis of Bovine Udder infection**. Arlington, VA, USA: National Mastitis Council, Inc.

HERTL J. A. *et al*. Pathogen-specific effects on milk yield in repeated clinical mastitis episodes in Holstein dairy cows. **J. Dairy Sci.,** Champaign, v. 97, n. 3, p. 1465-1480, 2014. DOI 10.3168/jds.2013-7266. Disponível em: https://www.sciencedirect.com/science/article/pii/S00 22030214000204. Acesso em: 14 dez. 2022.

JØRGENSEN *et al. Streptococcus agalactiae* in the environment of bovine dairy herds—rewriting the textbooks? **Vet. Microbiol.**, Amsterdam, v. 184, p. 64-72, 2016. DOI 10.106/j. vetmic.2015.12.014. Disponível em: https://www.sciencedirect.com/science/article/pii/S0378 113515301206. Acesso em: 10 mai. 2023.

KOIVULA, M. *et al*. Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland. **Acta Agric. Scand. A. Anim. Sci.**, Copenhagen, v. 57, n. 2, p. 89-96, 2007. DOI 10.1080/09064700701488941. Disponível em: https://www. tandfonline.com/doi/full/10.1080/09064700701488941. Acesso em: 15 out. 2020.

LANGONI, H. *et al*. Aspectos microbiológicos e de qualidade do leite bovino. **Pesq. Vet. Bras.**, Rio de Janeiro, v: 31, n 12, p. 1059-1065, 2011. DOI 10.159/S0100736X201100 1200004. Disponível em: https://www.scielo.br/j/pvb/a/WBGK65gdTzpdrtLSCk3TY9M/ abstract/?lang= en. Acesso em: 10 abr. 2023.

LUCKEN, A. *et al*. Intramammary infections with Corynebacterium spp. in bovine lactating udder quarters. **PLoS ONE** v. 17, n. 7, p. 1-13, 2022. DOI 10.1371/journal.pone.0270867. Disponível em: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0270867. Acesso em: 11 mai. 2023.

MAKOVEC, J. A.; RUEGG, P. L. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. **J. Dairy Sci.**, Champaign, v. 86, n. 1 , p. 3466–3472, 2003. DOI 10.3168/jds.S0022-0302(03)73951-4. Disponível em: https://www.science direct.com/science/article/pii/S0022030203739514. Acesso em: 15 out. 2020.

NAHMS. 2021. Dairy 2014. Trends in Dairy Cattle Health and Management Practices in the United States, 1991-2014. Disponível em: https://www.aphis.usda.gov/aphis/ourfocus%20/% 20%20animalhealth/monitoring-and-surveillance/nahms/nahms-studies/nahms-studies-table. Acesso em: 25 nov. 2022.

ØSTERÅ, O. *et al*. Milk culture results in a large Norwegian survey-effects of season, parity, days in milk, resistance, and clustering. **J. Dairy Sci.**, Champaign, v. 89, n. 3, p.1010-1023, 2006. DOI 10. 3168/jds.S0022-0302(06)72167-1. Disponível em: https://www.sciencedirect. com/science/article/pii/S0022030206721671. Acesso em: 21 out. 2019.

PANKEY, J. W., *et al*. Effects of *Corynebacterium bovis* infection on susceptibility to major mastitis pathogens. **J. Dairy Sci.,** Champaign, v. 68, n. 10, p. 2684-2693, 1985. DOI 10.3168/jds.S0022-0302(85)81153-X. Disponível em: https://pubmed.ncbi.nlm.nih.gov/390 5890/. Acesso em: 14 dez. 2022.

PIEPERS, S. *et al*. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. **J. Dairy Res.**, London, v. 74, n. 4, p. 478–483, 2007. DOI 10.1017/s0022029937002841. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/ S0022030284815921. Acesso em: 10 out. 2020.

RODRIGUES, A. C. O.; RUEGG, P. L. Actions and outcomes of Wisconsin dairy farms completing milk quality teams. **J. Dairy Sci.**, Champaign, v. 88, n. 7 , p. 2672-2680, 2005. DOI 10.3168/jds.S0022-0302(05)72944-1. Disponível em: https://pubmed.ncbi.nlm.nih. gov/15956329. Acesso em: 28 out. 2019.

ROSSITTO, P. V. *et al*. Antibiotic susceptibility patterns for environmental *Streptococci* isolated from bovine mastitis in central California dairies. **J. Dairy Sci.**, Champaign, v. 85, n. 1, p. 132-138, 2002. DOI 10.3168/jds.S0022-0302(02)74061-7. Disponível em: https:// pubmed.ncbi.nlm.nih.gov/11860105. Acesso em: 24 abr. 2023.

RUEGG, P. L. Investigation of mastitis problems on farms. **Vet. Clin. North Am. Food Anim. Pract.**, Philadelphia, v. 19, n. 1, p. 47-73, 2003. DOI 10.1016/S0749-0720(02)00078-6. Disponível em: https://www.sciencedirect.com/science/article/pii/S0022030217310329. Acesso em: 16 ago. 2020.

RUEGG, P. L. A 100-Year Review: Mastitis detection, management, and prevention. **J**. **Dairy Sci.**, Champaign, v. 100, n. 12, p. 10381–10397, 2017. DOI 10.3168/jds.2017-13023. Disponível em: https://www.sciencedirect.com/science/article/pii/S0022030217310329. Acesso em: 10 mar. 2019.

SANTOS, J. E. P. *et al*. Effect of timing of first clinical mastitis occurrence on lactational and reproductive performance of Holstein dairy cows. **Anim. Reprod. Sci.,** Amsterdam, v. 80, n. 1-2, p. 31–45, 2004. DOI 10.1016/S0378-4320(03)00133-7. Disponível em: https://pubmed. ncbi.nlm.nih.gov/15036513. Acesso em: 21 abr. 2022.

SCHRICK, F. N. *et al*. Influence of subclinical mastitis during early lactation on reproductive parameters. **J. Dairy Sci.,** Champaign, v. 84, n. 6, 1407-1412, 2001. DOI 10.3168/jds.S0022-0302(01)70172-5. Disponível em: https://pubmed.ncbi.nlm.nih.gov/11417699. Acesso em: 21 abr. 2022.

SMITH, K. L. *et al*. Environmental mastitis: cause, prevalence, prevention. **J. Dairy Sci.**, Champaign, v. 68, n. 6 p. 1531-1553, 1985. DOI 10.3168/jds.S0022-0302(85)80993-0. Disponível em: https://pubmed.ncbi.nlm.nih.gov/4019890. Acesso em: 30 nov. 2022.

TENHAGEN, B. A. *et al*. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. **J. Dairy Sci**., Champaign, v. 89, n. 7, p. 2542–2551, 2006. DOI 10.3168/jds.S0022-0302(06)72330-X. Disponível em: https://linkinghub.elsevier.com/retrieve/pii/S002203020672330X. Acesso em: 26 ago. 2020.

TOMAZI, T. *et al*. Association of herd-level risk factors and incidence rate of clinical mastitis in 20 Brazilian dairy herds. **Prev. Vet. Med.**, Amsterdam, v. 161, p. 9–18, 2018. DOI 10.1016/j.prevetmed.2018.10.007. Disponível em: https://www.sciencedirect.com/science/article/pii/S0167587718302691. Acesso em: 18 ago. 2020.

WATTS, J. L. Etiological agents of bovine mastitis. **Vet. Microbiol**., Amsterdam, v. 16, n. 1, p. 41-66, 1988. DOI 10.1016/0378-1135(88)90126-5. Disponível em: https://pubmed.ncbi.nlm.nih.gov/3354192/. Acesso em: 09 mar. 2020.

**CAPÍTULO 2**

**Dynamics of the prevalence of mastitis-causing pathogens in Brazilian dairy farms from 2012 and 2020 and association with year and season**

**INTERPRETATIVE SUMMARY**

**Dynamics of mastitis-causing pathogens prevalence in Brazilian dairy farms from 2012 to 2020 and association with year and season.** *By Sekito et al.* Mastitis is caused by a diversity of microorganisms and remains an important disease causing economic losses to the dairy industry. Here we characterized the microbial profile in 717,168 milk samples submitted to a diagnostic laboratory from 3,793 Brazilian dairy farms from 2012 to 2020. The results showed a small reduction in the prevalence of contagious mastitis pathogens overtime, but a subset of farms committed to the identification of mastitis-causing pathogens substantially reduced the prevalence of *Streptococcus agalactiae* from their herds.

Running head: MASTITIS PATHOGENS IN BRAZILIAN DAIRY FARMS

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**ABSTRACT**

Objectives were to determine the profile and prevalence of contagious and environmental mastitis-causing pathogens in milk samples from cows experiencing clinical or subclinical mastitis, or from udder health monitoring programs submitted to a commercial diagnostic laboratory over a period of 9 years. This observational longitudinal study analyzed the microbiological profile from 717,168 milk samples from 3,793 dairy farms from 2012 to 2020. Further analyses were performed in a subset of 204,461 microbiological results of 12 farms that submitted samples in all 9 years. Milk samples originated from individual quarters or composite samples. A growth result was defined when 3 or more colonies from 1 to 3 different pathogens were present in culture media, except for *Staphylococcus aureus* and *Streptococcus agalactiae* which 1 colony was defined as growth. A contaminated result was defined when 1 or more colonies from 4 or more microorganisms were present in culture media. In samples from the 3,793 farms, the prevalence of contagious mastitis-causing pathogens was 28.7% in 2012 and 26.6% in 2020, with summer having the greatest prevalence, 27.6%. The prevalence of environmental mastitis-causing pathogens was approximately 26.0% in 2012 and 2020, and fall and spring seasons had the greatest prevalence, 27.5%. The prevalence of *S. aureus* did not differ between 2012 and 2020, 10.9% and 10.3%, respectively, but season was associated with the prevalence. The prevalence of *S. agalactiae* decreased from 7.19% in 2012 to 3.54% in 2020, and winter had the greatest prevalence, 7.73%, compared with fall, spring, or summer. The most prevalent mastitis-causing pathogens were non-*aureus* staphylococci (NAS), *Staphylococcus chromogenes*, *S. aureus*, *S. agalactiae*, *Corynebacterium bovis*, *Klebsiella* spp, *Streptococcus uberis*, *Escherichia coli*, *Streptococcus dysgalactiae*, and *Pseudomonas* spp. The 12 farms that submitted milk samples in all 9 years had a reduction in the prevalence of contagious mastitis-causing pathogens from 15.1% in 2012 to 8.02% in 2020. Indeed, the prevalence of *S. agalactiae* decreased from 5.66% in 2013 to 0.21% in 2020. Similarly, the prevalence of environmental mastitis-causing pathogens in milk samples decreased from 38.2% in 2012 to 25.1% in 2020,

with summer presenting the greatest prevalence, 34.5%. A small gradual reduction in the prevalence of contagious mastitis-causing pathogens was observed in dairy farms in Brazil, but a change in the profile from contagious to environmental pathogens was not detected in the 3,793 farms. Conversely, the subset of farms committed to the identification of mastitis-causing pathogens had a reduction in the prevalence of *S. agalactiae* and experienced a change in the profile towards environmental mastitis-causing pathogens. Producers committed to the identification of mastitis-causing pathogens are likely to implement specific control measures to reduce the prevalence of contagious bacteria that cause mastitis.

**Key words**: contagious, environmental, mastitis, microorganisms, prevalence

## INTRODUCTION

Mastitis remains one of the main diseases affecting dairy cows (NAHMS, 2021), and it is associated with negative impacts on milk production, reproduction, and survival of cows (Schrick et al., 2001; Santos et al., 2004). Such impacts lead to major economic losses to the dairy industry, including treatment costs, discarded milk, increased culling, increased labor, and decreased milk quality (DeGraves and Fetrow, 1993; Aghamohammadi et al., 2018). Mastitis has a diverse etiology, often associated with bacterial pathogens (Watts, 1988), of which some are highly prevalent with specific microbiological features (Smith et al., 1985; Fox and Gay, 1993). In general, contagious mastitis-causing pathogens reside in the cow's teat skin or the mammary gland, and transmission among cows occurs during milking (Fox and Gay, 1993), whereas environmental pathogens reside in the cow's environment, and the contamination of the teat occurs between milkings which increases the risk of these microorganisms entering the teat cistern and eventually accessing the mammary gland tissue during the milking process (Watts, 1988). Understanding the agent of mastitis allows for the implementation of specific control measures related to therapy and prevention of mastitis (Rossitto et al., 2002; Dohoo et al., 2011).

Herds or countries that consistently adopted prevention strategies and target therapy have successfully controlled or eradicated contagious pathogens of mastitis such as *Streptococcus*

*agalactiae*, *Staphylococcus aureus*, and *Corynebacterium bovis* (Makovec and Ruegg, 2003; Koivula et al., 2007; Piepers et al., 2007; Dyson et al., 2022). These prevention measures have resulted in a change over time in the profile of mastitis-causing pathogens from contagious to environmental within well-managed herds (DeGraves and Fetrow, 1993; Ruegg, 2017). In Brazil, studies have estimated the prevalence of *S. agalactiae* ranging from 7.0% to 16.9%, *S. aureus* from 9.6% to 19.2%, and *Corynebacterium* spp from 9.0% to 55.2% (Brito et al., 1999; Langoni et al., 2011; Barbosa et al., 2017; Tomazi et al., 2018). Nevertheless, no national surveillance system exists in Brazil for monitoring mastitis pathogens in the dairy herds.

The present longitudinal study covers diverse areas of milk production in Brazil, including samples from the 5 geographical regions of the country during the 4 seasons of the year, thus providing the opportunity to characterize the bacterial etiology of bovine mastitis in Brazilian dairy herds that represents the status of the dairy herd in the country during the period studied.

We hypothesized that the prevalence of contagious and environmental mastitis pathogens in Brazilian dairy herds remains relevant and that the change from contagious to environmental mastitis profile has not yet occurred as observed in some countries. This longitudinal study aimed to determine, over a period of 9 years, the dynamics of the prevalence and profile of mastitis-causing pathogens isolated from milk samples from cows experiencing mastitis or from udder health monitoring programs submitted to a commercial diagnostic laboratory and their association with year and season.

## MATERIALS AND METHODS

The Vidavet Veterinary Analysis Laboratory (https://www.labvidavet.com/), a commercial diagnostic laboratory, authorized for research purposes the access and use of its database with all microbiological results of milk samples analyzed from 2012 to 2020. The Vidavet Laboratory obtained ISO/IEC 17025:2005 accreditation, Proficiency Testing Certification, and it was regularly inspected by regional authorities such as the Brazilian Ministry of Agriculture (MAPA) during the entire period of the study.

This observational study was based only on detailed records of microbiological reports, and no animal or human intervention was performed. Therefore, ethical approval for use of animals and human subjects are not applicable.

### *Study Design*

This longitudinal observational study was based on a convenience database with microbiological results of milk samples collected from dairy cows naturally exposed to mastitis-causing pathogens and submitted to the diagnostic laboratory. This convenience database from one diagnostic laboratory likely reduced potential biases that could be attributed to differences in laboratory procedures. Procedures used for culturing milk remained consistent throughout the study period. The study was reported according to The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (Sargeant and O'Connor, 2014).

### *Milk Samples*

Milk samples originated from individual quarters or composite collection from cows experiencing clinical or subclinical mastitis, or cows subjected to udder health monitoring programs from dairy farms located in all 5 regions in Brazil, namely Central West, North, Northeast, South, and Southeast.

### *Microbiological Culture*

The sampling guidelines provided to all producers by the laboratory included that individual teats be immersed and sanitized in a pre-dipping solution before milking. After 30 seconds of contact with the pre-dipping solution, the teat should be dried, and the first 3 strips of milk be discarded. Then, removal of any debris and cleaning of the teat end should be performed using gauze embedded in 70% alcohol. Wearing gloves, the person should take an aseptic milk sample into a properly identified sterile plastic tube to be refrigerated or frozen immediately after sampling. The refrigerated or frozen sample should be properly packaged, identified, and shipped in thermal boxes to the laboratory at the farm's earliest convenience.

Upon arrival at the laboratory, the samples were thawed at room temperature and cultured according to standard microbiological methods from National Mastitis Council (Hogan et al., 2005). Briefly, each sample was swirled and approximately 10 μL of milk was streaked onto bovine blood agar and MacConkey plates and incubated under aerobic conditions at 35°C to 37°C for 48 hours. The technician examined the plates for growth at 24 and 48 hours and performed the identification of the isolate based on colony morphology, hemolysis, Gram staining, microscopic appearance after staining, catalase, and biochemical testing (Holt et al., 1994; Garrity and Holt, 2001a; Hogan et al., 2005).

Staphylococci were identified using mannitol, coagulase, DNase activity, Baird Parker agar (HiMedia Laboratories LLC), pyrrolidonyl arilamidase, brain and heart infusion broth, and Muller Hinton for bacitracin, polymyxin and novobiocin tests (ANVISA, 2013), and sugar fermentation profiles. Streptococci, enterococci, and lactococci were identified using the CAMP esculin test and growth on bile esculin, NaCl, pyrrolidonyl arilamidase, brain and heart infusion broth, and Muller Hinton agar for sulfa disc test, and bacitracin test (ANVISA, 2013), and sugar fermentation profiles. For the identification of *Corynebacterium* spp and *Trueperella pyogenes,* lactophenol blue, hemolysis, catalase, urea, and esculin tests were used. Catalase, oxidase, and KOH identified Gram-negative bacteria. For coliform identification, the Probac EPM Mili kit test (Probac do Brasil Bacteriological Products Ltda.) and Simmons citrate were used. *Pseudomonas* spp was identified by the Probac NF III kit test (Probac do Brasil Bacteriological Products Ltda.) and motility. *Pasteurella* spp was identified using indole. *Bacillus* spp and *Nocardia* spp were identified by Gram staining and morphology. *Prototheca* spp, fungi, and yeasts were identified by lactophenol blue and Sabouraud media with chloramphenicol.

### Definitions of Microbiological Results

The culture results were initially categorized into no growth, contaminated, and growth, regardless of whether the sample originated from quarters or from a composite collection. The contaminated result was defined as the growth of at least one colony from four or more different

microorganisms in the culture media. The growth result was defined as the presence of three or more colonies for up to three microorganisms, except for *S. agalactiae* and *S. aureus* which a single colony was defined as a growth result. No growth result was defined when none of the above conditions were observed. Non-*aureus* staphylococci (**NAS**) included *S. chromogenes*, *S. cohnii*, *S. epidermidis, S. equorum*, *S. haemolyticus*, *S. hyicus*, *S. intermedius*, *S. saprophyticus*, *S. sciuri*, *S. simulans*, *S. warneri*, and *S. xylosus*, all of which were isolated in milk samples in the study. Isolation of *S. agalactiae*, *S. aureus*, *C. bovis,* and *Corynebacterium* spp were classified as contagious pathogens, whereas coliforms, environmental streptococci, enterococci, lactococci, *Pseudomonas* spp, *S. cohnii, S. equorum, S. haemolyticus, S. saprophyticus, S. simulans*, and *S. sciuri* were considered environmental pathogens. Other NAS such as *S. chromogenes*, *S. epidermidis*, *S. hyicus*, *S. intermedius*, *S. warneri*, and *S. xylosus* were not grouped as neither contagious nor environmental because of lack of agreement in the literature (Piessens et al., 2011, De Visscher et al., 2016, Jenkins et al., 2019, De Buck et al., 2021). An individual sample that had more than one microorganism isolated could be classified as being part of more than one group of mastitis pathogens. Similarly, a milk sample with isolation of more than one microorganism, for instance *S. aureus* and *S. chromogenes*, was considered positive for both microorganisms for the analysis of individual pathogens in milk samples.

### *Seasons of the Year*

Seasons were defined as fall from March 20th to June 20th, winter from June 21st to September 21st, spring from September 22nd to December 20th, and summer from December 21st to March 19th, for all years and based on the arrival date of the milk samples at the laboratory.

### *Preparation of the Database*

The microbiological results were recorded into spreadsheets (Microsoft Excel, Microsoft Corporation 2022) and included the sample identification, the farm and owner′s identification and geographic location, the date of sample collection, the date of arrival in the laboratory, and the microbiological results. The spreadsheets were processed, and data were retrieved using the

Visual Basic application (Microsoft Corporation 2022) into one file. During the process, the data were cleaned, reviewed and prepared for statistical analyses. The authors identified 3,793 farms that submitted milk samples to the laboratory during the study period. Of those, 12 farms submitted milk samples in all 9 years of the study. These 12 farms generated a subset of the data.

### Exclusion Criteria

Microbiological results were ineligible for analyses if they were replicated on the database, whenever not submitted by a private farm, when no sample identification was provided, or if the milk sample did not arrive at the laboratory refrigerated.

### Responses Analyzed

Microbiological results were analyzed either as individual isolates (e.g., *S. chromogenes*, *S. aureus*, *S. agalactiae*, etc.) or as a group of mastitis pathogens such as those identified as NAS, contagious, or environmental pathogens. Responses were analyzed for all 3,793 farms or for the subset of 12 farms. Responses analyzed included: a) proportion of farms that had at least one positive microbiological result for a group of pathogens or for a specified mastitis pathogen relative to all farms that submitted samples; b) proportion of milk samples with no growth, growth or contaminated results relative to all milk samples; c) proportion of individual pathogens or groups of pathogens (NAS, contagious, environmental) isolated from milk samples, but excluding contaminated samples; and d) proportion of individual pathogens or group of pathogens (NAS) isolated from milk samples according to type of mastitis identified, but excluding the contaminated samples.

### Statistical Analyses

Binary data were analyzed by logistic regression using generalized linear models with the GLIMMIX procedure of SAS (SAS/STAT, SAS Institute Inc.) fitting a binary distribution with either only fixed or with mixed effects. Data analyzed included the probability of isolation of individual pathogens (e.g., *S. agalactiae, S. aureus*, *S. chromogenes*, etc.) or groups of pathogens (e.g., contagious, environmental, NAS). For the 3,793 farms, the statistical models included the

fixed effects of region (Central West, North and Northeast combined, South, and Southeast), season of the year (fall, winter, spring and summer), year of submission (2012 to 2020), and the interactions between region and season, region and year, and season and year. For the data from the 12 farms, the statistical models included the fixed effects of region (South and Southeast), season of the year (fall, winter, spring and summer), year of submission (2012 to 2020), and the interactions between region and season, region and year, and season and year, and the random effect of farm. For the subset of samples with identification of clinical or subclinical mastitis, the association between type of mastitis and the isolation of the most prevalent pathogens in milk was analyzed for the 3,793 and 12 farms with generalized linear mixed-effects models. The models included the fixed effects of type of mastitis (clinical vs. subclinical), year of submission, and season, and the random effect of farm.

In all statistical models, the Kenward-Roger method was used to approximate the denominator degrees of freedom to compute the $F$-tests. The adjusted odds ratios (**OR**) and 95% confidence intervals (**CI**) were also computed. Data are presented as least-squares means (**LSM**) and standard errors of the means (**SEM**), and significance was declared when $P \leq 0.05$. Whenever significance against the null hypothesis was observed ($P \leq 0.05$), separation among LSM was performed after correction by the Tukey method for multiple comparisons.

## RESULTS

### Data from all 3,793 Farms

***Completeness of the database***. The initial database had a total of 817,038 milk samples with microbiological results. Figure 1 depicts the flow of data that met or did not meet the eligibility criteria. After exclusion, there were 717,168 microbiological results that remained in the study and were used for statistical analyses of no growth, contaminated, and growth results (Figure 1). Of those, 698,585 microbiological results had no information if the mastitis was clinical or subclinical, whereas 18,593 microbiological results originated from samples with identification of the type of mastitis, if clinical (15,991) or subclinical (2,592). For analysis of

groups of pathogens or specified pathogens, the contaminated results were excluded, and the remaining 679,706 microbiological results were used (Figure 1).

*Descriptive analysis of the microbiological results*. Approximately 7.9% (n = 56,702) of the samples originated from the Central West region, 1.7% (n = 11,953) from the North and Northeast regions combined, 5.6% (n = 40,082) from the South, 79.4% (n = 569,491) from the Southeast region, and 5.4% (n = 38,940) from unidentified region in Brazil. There were 37.1% (n = 266,248) no growth, 5.22% (n = 37,462) contaminated, and 57.7% (n = 413,458) growth results. In total, 55 pathogens were isolated from milk, of which 82.3% (n = 340,393) were pure culture, 16.4% (n = 67,891) had 2 isolated pathogens, and 1.3% (n = 5,174) had 3 isolated pathogens.

Table 1 depicts the proportion of farms that presented at least one positive microbiological result in the first half, from 2012 to July 2016 (1,858 farms), and the second half of the study period, from August 2016 to 2020 (2,399 farms). Contagious pathogens were isolated from milk samples from most farms, and only a small reduction of 4.7-percentage units was observed between the first and the second half period of the study. Within contagious pathogens, *S. aureus* and *S. agalactiae* contributed with most of the reduction in farms with positive isolates, 9.10- and 12.6-percentage units decrease, respectively (Table 1). Isolation of environmental pathogens presented a small increase of 2.20-percentage units between the two periods of the study. This increase was caused by *S. uberis* and *S. dysgalactiae* which increased 16.4- and 4.50-percentage units. Conversely, *Klebsiella* spp and *E. coli* presented the greatest decrease, 11.0- and 12.2-percentage units, respectively. The NAS and *S. chromogenes* were isolated from milk samples from the majority of farms, with increases of 3.40- and 10.4-percentage units, respectively, between the first and second half of the study (Table 1).

*Results according to the type of mastitis*. From 2012 to 2018, 92% of 18,583 samples with identification of the type of mastitis originated from clinical cases and only 8% for subclinical

mastitis cases; however, these proportions shifted in the last 2 years of the study when 51% of the samples originated from clinical mastitis cases and 49% from subclinical cases (Figure 2A).

***The most prevalent pathogens in milk samples.*** *Staphylococcus chromogenes*, *S. aureus*, *S. agalactiae*, *C. bovis*, *Klebsiella* spp, *S. uberis*, *E. coli*, *S. dysgalactiae and Pseudomonas* spp were the most frequently isolated specified pathogens in the study (Table 2). Because of the high prevalence of samples with *S. chromogenes*, the NAS group was the most prevalent in the study. Of the 18,583 samples with identification of type of mastitis, 3.45% (n = 641) were contaminated and not included in further statistical analyses. Of the non-contaminated samples, NAS, *S. chromogenes* and *C. bovis* were more frequently ($P < 0.001$) isolated from subclinical than clinical mastitis samples, whereas *S. agalactiae*, *Klebsiella* spp, *S. uberis*, and *E. coli* were more ($P < 0.001$) frequently isolated from clinical than subclinical mastitis cases (Table 3). No difference in isolation was observed for *S. aureus*, *S. dysgalactiae* and *Pseudomonas* spp from milk samples originated from clinical and subclinical mastitis cases.

***Microbiological prevalence and associations with year and season***. Year, season, and the interaction of year and season were associated ($P < 0.001$) with multiple culture results analyzed (Table 4; Figure 3A). No growth represented 37.1% of the samples and the prevalence decreased ($P < 0.001$) from 2012 to 2020. Winter had the greatest ($P < 0.001$) prevalence of no growth followed by summer, fall and spring (Figure 3B). Approximately 5.22% of the samples were contaminated and the prevalence increased ($P < 0.001$) between 2012 and 2020 (Table 4; Figure 3A). Spring had the greatest prevalence and differed ($P \leq 0.05$) from fall, winter and summer (Figure 3C). Growth represented 57.7% of all samples and the prevalence was associated ($P < 0.001$) with year, but not with season (Table 4; Figures 3A and 3D).

The prevalence of contagious pathogens isolated from milk samples presented a small decrease ($P < 0.001$) from 2012 to 2018, namely *S. aureus, S. agalactiae, and C. bovis* but, except for *S. agalactiae*, isolation of those contagious pathogens from samples submitted in 2020 returned to similar values as observed in 2012 (Table 4; Figure 4A). Summer was associated

with the greatest ($P \leq 0.05$) prevalence of contagious pathogens compared to other seasons (Figure 4B). The prevalence of *S. agalactiae* presented a small gradual reduction ($P = 0.01$) from 2012 to 2020, and winter had greater ($P < 0.001$) prevalence compared to fall, spring, and summer (Figure 5C). The prevalence of *S. aureus* varied across the study period, and a small increase ($P \leq 0.05$) was observed in samples submitted during the summer months compared with the other seasons of the year (Figure 5A). Similar to *S. aureus*, the prevalence of *C. bovis* in milk varied over the years (Table 4), with no difference for years 2012 and 2020, and samples submitted in the summer had greater ($P \leq 0.05$) prevalence than winter and spring (Figure 5D).

Environmental pathogens were isolated from approximately 27% of the milk samples during the study period, although, the greatest ($P < 0.001$) prevalence was observed in samples from 2018 (Table 4; Figure 4C). Fall and spring had greater prevalence ($P < 0.001$) compared to winter and summer (Figure 4D). The most prevalent pathogens isolated from milk samples was the group of NAS, and isolation of NAS and *S. chromogenes* increased ($P < 0.001$) 11.0- and 14.6-percentage units, respectively from 2012 to 2020 (Table 4). *Klebsiella* spp and *E. coli* showed the greatest reduction ($P < 0.001$) in isolation over the years, conversely isolation of *S. uberis* increased ($P < 0.001$) during the study period. Isolation of *S. dysgalactiae* and *Pseudomonas* spp varied over the years, but the differences were of small magnitude (Table 4).

*** Subset of 12 Farms ***

***Completeness of the database.*** The flow of data from samples originating from the 12 farms is depicted in Figure 1. For the analysis of no growth, contaminated, and growth results, 204,461 microbiological results were considered. After exclusion of contaminated results, there were 193,914 microbiological results used for statistical analysis for group or specified pathogens results (Figure 1). Of those, 182,909 microbiological results had no information related to the type of mastitis, whereas 11,005 microbiological results originated from samples with indication of the type of mastitis, if clinical (9,434) or subclinical (1,571).

***Descriptive analysis of the microbiological results***. Of the 204,461 microbiological results from the 12 farms, 14.4% (29,497) originated from 2 farms located in the South, and 85.6% (174,964) from the remaining 10 farms located in the Southeast region of Brazil. There were 43.2% (88,425) no growth results, 5.2% (10,547) contaminated results, and 51.6% (105,489) growth results. In total, 46 pathogens were isolated from the 105,489 growth results, of which 83.3% (87,838) were pure culture, 15.5% (16,370) with 2 isolated pathogens, and 1.2% (1,281) with 3 isolated pathogens in culture media.

***Results according to the type of mastitis.*** Of the 11,005 milk samples with identified types of mastitis, 2012 has primarily subclinical samples and 2013 primarily clinical samples. From 2014 to 2018, most submitted samples were from clinical mastitis cases, whereas in the last 2 years, 2019 and 2020, the proportions of clinical samples submitted decreased with the increase in subclinical samples (Figure 2B). The NAS, *S. chromogenes*, and *C. bovis* were more frequently ($P < 0.001$) isolated from subclinical compared with clinical mastitis samples (Table 3). On the other hand, *S. agalactiae, Klebsiella* spp, *S. uberis*, and *E. coli* were more frequently ($P < 0.001$) isolated from clinical compared with subclinical mastitis samples. No difference was observed for isolation of *S. aureus, S. dysgalactiae* and *Pseudomonas* spp between clinical and subclinical samples.

***Microbiological prevalence and association with year and season***. Year, season, and interaction between year and season were associated ($P < 0.001$) with multiple culture results analyzed (Table 5; Figures 6-8). The prevalence of samples with no growth was associated ($P < 0.001$) with year, although they did not differ between the first and the last year of the study (Table 5; Figure 6A). Milk samples with no growth had greater ($P < 0.05$) prevalence in the winter season, followed by fall and spring, and was least during the summer season (Figure 6B). Although the proportion of contaminated milk samples was small, it increased ($P < 0.001$) from 2012 to 2020 and it was greater ($P < 0.05$) in the fall, followed by spring and summer, and least frequent in the winter months (Figure 6C). Growth results decreased ($P < 0.001$) during the study

period (Figure 6A), with summer having greater ($P < 0.05$) proportion of samples with growth, followed by fall and spring, and then winter (Figure 6D).

Milk samples with isolation of contagious pathogens decreased ($P < 0.001$) over the course of the study (Table 5; Figure 7A) and it was greatest during the summer ($P < 0.001$) than winter and spring months (Figure 7B). All 3 contagious pathogens, *S. aureus*, *C. bovis* and *S. agalactiae* decreased ($P < 0.001$) over time, except in 2020 when the proportion of samples with isolated *C. bovis* increased relative to the previous year (Figure 8A). Season was not associated with *S. aureus*, but milk samples in which *S. agalactiae* was isolated was greater ($P < 0.01$) in the summer and winter than fall and spring, whereas for *C. bovis*, isolation was more common ($P < 0.01$) in summer and fall than winter and spring (Figures 8B-8D).

The pattern of isolation of environmental pathogens was variable over the course of the study, but it decreased ($P < 0.01$) 13-percentage units from 2012 to 2020 (Table 5, Figure 7C). Summer was associated with the greatest and winter had the smallest prevalence of environmental pathogens isolated from milk (Figure 7D). The most common group of pathogens isolated from milk samples was NAS and their prevalence increased ($P < 0.001$) 26.9-percentage units from 2012 to 2020. Within this group, *S. chromogenes* was the most prevalent and, as expected, isolation from milk samples increased ($P < 0.001$) 25-percentage units in the study period (Table 5). Isolation of *Klebsiella* spp and *E. coli* decreased ($P < 0.001$) during the study, but those of *S. uberis, S. dysgalactiae* and *Pseudomonas* spp, although affected by year, minor differences were observed between the first and last years of the study (Table 5).

## DISCUSSION

Contagious pathogens remain highly prevalent in milk samples from mastitis cases submitted for microbiological culture in Brazil, and a large proportion of Brazilian dairy herds have cows harboring contagious pathogens. In the present study, milk samples from 3,793 farms cultured under standard microbiological specifications showed only a small decrease in the proportion of samples in which contagious pathogens were isolated between 2012 and 2020. The

small decrease was observed because of a reduction in isolation of *S. agalactiae*, whereas isolation of *S. aureus* and *C. bovis* varied over the years, but showed no clear indication of a reduction in prevalence in cultured milk samples between 2012 and 2020. On the other hand, data from the 12 farms that submitted samples every year showed a continuous reduction in all 3 contagious pathogens, in particular *S. agalactiae*, likely because they were more committed to controlling those agents. The most prevalent group of pathogens and individual pathogen in our database were NAS and *S. chromogenes* that presented a substantial gradual increase during the 9 years and this pattern of isolated pathogens was observed in the samples submitted by all farms as well as in the samples from the 12 farms.

### *Data From the 3,793 Farms*

Isolation of no pathogen from milk samples occurred in similar frequency to those reported by other studies (Makovec and Ruegg, 2003; Koivula et al., 2007; Dyson et al., 2022). No growth is often attributed to resolution of the mastitis case in which the immune response of the cow either completely eliminates the pathogen or reduces the pathogen load to a concentration in milk that is below the detection limit of the culture method (Santos et al., 2004). Also, freezing of samples might kill some of the pathogens (Schukken et al., 1989), thus reducing the ability of the culture system to detect growth. Finally, the culture system used was not specific for some pathogens that require specific media and culture conditions such as *Mycoplasma* spp, which could be present but remained not detected because of the methods used. Regardless, a relatively large proportion of submitted milk samples resulted in no growth as reported elsewhere (Oliveira et al., 2013). Negative growth results were more common during the winter season. Others have observed that no growth also was associated with season, but they were more likely to occur either in the spring (Makovec and Ruegg, 2003) or summer (Koivula et al., 2007).

The proportion of contaminated samples varied and increased over time reaching 7% of the samples submitted for culture in 2020. The definition of a contaminated sample in the current study was less restrictive compared to other studies in which samples with 2 or more or 3 or

more isolates from a single sample were classified as contaminated (Ferguson et al., 2007, Gao et al., 2017, Dyson et al., 2022). Contaminated samples are likely the result of inadequate care during cow preparation before sampling and most likely reflect bacterial present on the surface of the skin of the teat of inadequate asepsis of the teat end.  In any case, the proportion of milk samples with 3 isolated microorganisms in the present study was small and it would have only a small impact on the results. Winter was the season associated with the smallest prevalence of contaminated samples, whereas spring with the greatest, which partially agrees with data from Makovec e Ruegg (2003) that observed that milk samples were less likely to be contaminated in the winter compared to summer and fall, or in the spring compared to summer or fall. Nevertheless, contaminated samples reflect inadequate cow preparation and reinforces the need for continuous training of personnel responsible for sampling, storage, and transportation of milk samples, especially in hot weather conditions.

Contagious pathogens showed a high prevalence of almost 30% of all samples submitted over the 9 years of the study, which is in contrast to countries or herds that consistently target the control or eradication of such mastitis pathogens (Makovec and Ruegg, 2003; Pitkälä et al., 2004; Piepers et al., 2007). The greatest prevalence was during the summer compared with all other seasons, although the magnitude of difference among seasons was small, statistically significant because of the very large sample size. Among the contagious pathogens we considered, both *S. aureus* and *C. bovis* showed almost no appreciable change over the 9-year study and over 10% of the samples were identified as having *S. aureus* or *C. bovis* in the first and the last year of the study period.

The observed prevalence of *S. aureus* agreed with that from other studies in Brazil, China and Australia (Langoni et al., 2011; Gao et al., 2017; Dyson et al., 2022). This major pathogen has specific characteristics imposing several predisposing factors to infection and chronicity, such as resistance to beta-lactam antibiotics, colonization of the teat skin, formation of micro-abscesses and biofilms, and mechanisms to evade the host's immune system (Fox and Gay, 1993;

Côte-Gravel and Malouin, 2019). Important measures to reduce the risk of *S. aureus* are proper milking procedures and culling of affected cows. We hypothesize that milking procedures were probably not adequate to contain the spread of *S. aureus* in those herds, but likely producers were not keen of culling cows because of mastitis. Although more than 3,793 producers submitted milk samples for culture, we hypothesize that most did not implement robust milk quality programs to control and/or eliminate contagious pathogens from their herds. Ferguson et al. (2007) observed that herds that enforced milk quality programs for more than 3 years had reduced the prevalence of contagious bacteria in milk samples. The relatively high prevalence of *S. aureus* in the samples studied and the specific microbiological feature of this pathogen suggest that more long-term effort is needed by Brazilian dairy producers to implement practices that control major pathogens, such as proper milking procedures, continued identification of infected animals, segregation during milking and culling of chronic animals. For instance, *C. bovis* is known to be controlled by proper milking procedures such as the use of pre- and post-dipping solutions with known killing activity against mastitis-causing pathogens (Foret et al., 2005). In the latter experiment, *C. bovis* was the most common agent of infection and authors justified that the agent is a common inhabitant of the ducts of the teats, thus requiring long-term continuous use of proper milking procedures that include dipping solutions to control the pathogen.

The prevalence of *C. bovis* in the first and last year of the study, 2012 and 2020, were greater than reported other studies in Norway, Belgium and Australia (Østerå et al., 2006; Piepers et al., 2007; Dyson et al., 2022). It is possible that almost 10% prevalence of *C. bovis* in milk samples cultured could be partially attributed to type of sample submitted, particularly originating from subclinical mastitis cases which had a 2.96 (95% CI = 2.34-3.75) times the odds to have *C. bovis* than clinical mastitis cases. Nevertheless, the proportion of samples isolated with *C. bovis* in the present study was comparable to that observed by Tomazi et al. (2018) also in Brazilian dairy farms. This minor pathogen has been associated with moderate increase in SCC and a reduction in the contents of lactose and non-fat milk solids (Schukken et al., 2009; Gonçalves et al., 2016).

In Finland, authors have reported increases in the prevalence of *C. bovis* from 16.6% in 1995 to 34.4% in 2001 and suggested that the low use of teat dipping solutions in Finnish dairy farms may have been the reason for such changes (Pitkälä et al., 2004).

A positive change observed in the present study was the reduction in prevalence of *S. agalactiae* over the 9-year period. However, one possibility is a shift in the proportion of clinical relative to subclinical milk samples submitted for culture by farmers. From the 18,583 milk samples identified according to the type of mastitis, there was a shift from clinical to subclinical samples in the last 2 years and the proportion of samples in which *S. agalactiae* was isolated markedly decreased in 2020. It is well known that *S. agalactiae* is less frequently isolated from subclinical than clinical cases of mastitis, a finding that was observed in the present study. The odds of isolating *S. agalactiae* was 10.4 (95% CI = 7.7-14.1) times greater in milk samples from clinical than subclinical mastitis cases. Another possibility is that producers implemented changes in management to control this pathogen. In any case, the prevalence of *S. agalactiae* in milk samples herein was similar to that observed in other studies reported in Brazil (Brito et al., 1999; Langoni et al., 2011; Tomazi et al., 2018). In contrast, *S. agalactiae* has been practically eradicated in many countries (Makovec and Ruegg, 2003; Østerå et al., 2006; Ferguson et al., 2007; Koivula et al., 2007; Dyson et al., 2022). Winter was the season of greatest prevalence of isolation of *S. agalactiae*, which also was observed by Makovec and Ruegg (2003). Nevertheless, although season was associated with *S. agalactiae*, the difference among winter and the season with the least prevalence, spring, was of only 1.3-percentage points, which may not be highly relevant. This pathogen is easily cultured from milk samples and responds well to antimicrobial therapy during lactation (Dinsmore et al., 1991; Fox and Gay, 1993), which makes eradication a common finding in numerous herds. Nevertheless, there has been recent evidence of the re-emergence of *S. agalactiae* in farms in Nordic countries, and Denmark reported an increase in prevalence to 5% in 2012 (Churakov et al., 2021). Jørgensen et al. (2016) suggested that *S. agalactiae* could be in the gastrointestinal tract and that an oral-fecal transmission via the

environment and water is possible. Also, Crestani et al. (2021) suggested that re-emergence of *S. agalactiae* could originate from transmission from humans to animals.

Of the pathogens defined among the group of environmental, *S. uberis* showed the greatest prevalence throughout the 9 years. Others have shown that *S. uberis* to be the most prevalent pathogen isolated from milk samples (Rossitto et al., 2002; Tomazi et al., 2018; Dyson et al., 2022). This bacterium is known to be more frequently isolated from milk at the end of lactation and from multiparous cows (Tenhagen et al., 2006). A variety of *S. uberis* strains is known to cause intramammary infections from environmental sources, but Zadocks et al. (2003) suggested that cows and milking machines are potentially the means of transmission. Thus, proper milking procedures are critical to control *S. uberis* in dairy farms.

Non-aureus staphylococci were the most predominant group of pathogens in the study, which agrees with studies worldwide (Tenhagen et al., 2006; Piepers et al., 2007; Bi et al., 2016; Dyson et al., 2022). Among them, *S. chromogenes* was the most frequently isolated from samples and the prevalence increased over the years. *Staphylococcus chromogenes* is a primary etiological agent of subclinical mastitis, although it can also cause clinical cases of mastitis, and it is associated with increased SCC and persistent intramammary infections (Condas et al., 2017; De Buck et al., 2021). Possibly, a shift in type of samples submitted could partially explain the change in profile of pathogens isolated from milk with the increase in NAS, in particular, *S. chromogenes* over the years. A gradual increase in the prevalence of *S. chromogenes* has also been observed by others (Makovec and Ruegg, 2003; Piepers et al., 2007; Schukken et al., 2009). Many NAS are considered to cause a minor or moderate increase in SCC (Schukken et al., 2009; Condas et al., 2017), and they are associated with little to no milk loss likely because of less udder damage compared to major pathogens (Hertl et al., 2014). It is unknown the mechanisms by which *S. chromogenes* and other NAS have become the dominate isolate in milk samples in numerous studies, but some suggest that *S. chromogenes* carry virulence factors such as exotoxin,

host evasion mechanisms, and ability to adhere to mammary epithelial cells that allow for the infection to persist compared to other NAS (De Buck et al., 2021).

Other environmental pathogens such as *E. coli* and *Klebsiella* spp presented a decrease in prevalence overtime, although other studies showed no reduction in frequency of those pathogens isolated from mastitis cases worldwide and in Brazil (Oliveira et al., 2013; Gao et al., 2016; Barbosa et al., 2017; Tomazi et al., 2018; Dalanezi et al., 2020). The lack of control of contagious pathogens, the incremental prevalence of NAS, and the shift in type of sample submitted from clinical to subclinical cases all might explain the decrease in frequency of isolation of *E. coli* and *Klebsiella* spp from 2012 to 2020 in Brazil (Piepers et al., 2007; Tomazi et al., 2018).

### *Data From the 12 Farms*

A large reduction, approximately 50%, in isolation of contagious pathogens was observed over the course of the study and this decrease was attributed to a large extent to the reduced prevalence of *S. agalactiae* that was almost not present in the last 2 years of the study in those 12 farms. Reductions were also observed for *S. aureus* and *C. bovis* overtime, thus resulting in a change from contagious to environmental pathogens that predominate in milk samples as observed in other studies (Makovec and Ruegg, 2003; Pitkälä et al., 2004; Piepers et al., 2007). The 12 farms consistently submitted milk samples for culture, and it is possible that they had greater commitment to implement specific measures of mastitis prevention, therapy, and eradication of certain pathogens control. Such results support the concept that farms that apply surveillance methods with continuous culture of milk samples from cases of clinical and subclinical mastitis are capable of controlling or even eradicating contagious pathogens from their herds. Summer was the season with the greatest prevalence of contagious pathogens compared to the other seasons. The magnitude of difference among seasons was small, although slightly larger than the magnitude observed in the 3,793 farms which may be attributed to the smaller sample size of the sub-dataset of 12 farms, still statistically significant.

The prevalence of samples with *S. aureus* isolated was comparable to that reported in Norway (Østerå et al., 2006). It is suggested that these 12 farms likely implemented measures such as proper milking procedures, culling of affected animals, and dry-cow therapy to control *S. aureus* in those herds. *Staphylococcus aureus* is often present in the skin of cattle and incoming first lactation cows can be a reservoir to increase the bacterial challenge in the herd, but the preventative practices during milking, dry-cow therapy, and selective culling are known to help control this pathogen by reducing new infections and eliminating the chronic cases (Ruegg, 2017). On the other hand, *S. agalactiae* has been eradicated from numerous herds in Europe (Østerå et al., 2006; Ferguson et al., 2007; Koivula et al., 2007), USA (Makovec and Ruegg, 2003) and Australia (Dyson et al., 2022). A similar pattern of reduction to almost elimination of *S. agalactiae* was observed in the samples from the 12 farms that resulted in only 0.21% prevalence in 2020. Obviously, one has to consider the type of samples submitted because *S. agalactiae* was more commonly isolated from clinical than subclinical cases and samples in 2020 were mostly from subclinical mastitis cases in this study.

Although environmental pathogens decreased over the years, *Klebsiella* spp and *E. coli* showed the greatest prevalence in most years, which agrees with data from other studies in Brazil and worldwide (Oliveira et al., 2013; Gao et al., 2016; Barbosa et al., 2017; Tomazi et al., 2018; Dalanezi et al., 2020) that reported the importance of these gram-negative pathogens as etiology of clinical mastitis.

### *Limitations and Potential Biases*

Herein we reported data from a diagnostic laboratory that analyzed milk samples submitted by commercial dairy farms. Farms submitted variable numbers of samples and most farms in the database did not submit milk samples in all 9 years, so the contributions of a given farm might weigh more heavily on a particular year and/or season of the year. An association exists between the type of mastitis and the pathogen isolated and only a fraction of the sample submitted had identified the type of mastitis, if clinical or subclinical. Thus, it is possible that changes in

patterns of pathogens over the years or seasons might reflect the type of sample submitted by producers. Similarly, milk samples were not identified if originating from individual quarters or composite of all quarters from a cow. It is known that some microorganisms, such as NAS, are isolated more frequently from composite than individual quarter samples or from nulliparous or primiparous cows (Ferguson et al., 2007; Reyher and Dohoo, 2011; De Buck et al., 2021). Interactions between year and season, region and year, and region and season were expected given the extremely large sample size; however, those interactions were not presented because of their complexity that would detract from the main objectives of the study, which were to evaluate the patterns of microbiological isolates from milk samples over time and among seasons. The author opted for significance combined with biological relevance to present and discuss the data (Chakkera et al., 2016). The study identified dynamics in the prevalence of group or specified pathogens isolated from milk samples throughout the 9 years; however, other factors such as management practices would need to be considered to better explain the observed changes overtime or among seasons.

## CONCLUSIONS

Understanding the profile of pathogens and its changes over time is important to guide farmers to better define the strategies of control, prevention, and treatment for mastitis that address the different pathogens with distinctive microbiological features in the dairy herds. A small reduction in the prevalence of pathogens of mastitis considered contagious was observed in farms in Brazil; however, a change in the profile from contagious towards environmental mastitis-causing pathogens was not identified in the 9 years in the 3,793 farms. In a subset of 12 farms that submitted samples in all 9 years, a clear reduction in the prevalence of milk samples with contagious pathogens was observed, in particular *S. agalactiae*, and the change from contagious to environmental mastitis-causing pathogens profile was observed. Mastitis remains relevant to the dairy industry and producers that are long-term committed to the identification of specific pathogens are those more likely to reduce their prevalence on the herd.

## AUTHORS' CONTRIBUTIONS

Luciana Sekito was responsible for data collection, processing, validation, data analyses, writing, review and edition of this article. Carla G. C. Vasconcelos performed most of the microbiological culture analysis, partially funded the study and contributed to the writing of the manuscript. José Luiz M. Vasconcelos was responsible for the conceptualization and the study design. Carlos A. Zambelli contributed to the data processing and validation. José Eduardo P. Santos was responsible for conceptualization and the study design, data analyses, writing and editing the manuscript, and study supervision.

## CONFLICTS OF INTEREST

One of the authors, Carla G. C. Vasconcelos, is the owner of the diagnostic laboratory where the milk samples were analyzed. All efforts were taken to ensure the impartiality and scientific rigor during the study and data analysis. All other authors declare no conflict of interest.

## ACKNOWLEDGMENTS

# REFERENCES

Aghamohammadi, M., D. Haine, D. F. Kelton, H. W. Barkema, H. Hogeveen, G. P. Keefe, and S. Dufour. 2018. Herd-level mastitis associated costs on Canadian dairy farms. Front. Vet. Sci. 5:1-12. https://doi.org/10.3389/fvets.2018.00100.

Barbosa, L. F. S. P., W. V. C. Oliveira, M. H. C. Pereira, M. B. Moreira, C. G. C. Vasconcelos, B. F. Silper, R. L. A. Cerri, and J. L. M. Vasconcelos. 2017. Somatic cell count and type of intramammary infection impacts fertility from *in vitro* produced embryo transfer. Theriogenology 108:291-296. https://doi.org/10.1016/j.theriogenology.2017.12.025.

Bi, Y., Y. J. Wang, Y. Qin, R. G. Valldervú, J. M. García, W. Sun, S. Li, and Z. Cao. 2016. Prevalence of Bovine Mastitis Pathogens in Bulk Tank Milk in China. PLoS ONE 11:1-13. https://doi.org/10.1371/journal.pone.0155621.

ANVISA. Agência Nacional de Vigilância Sanitária. Brazil. 2013. Módulo 6: Detecção e Identificação de Bactérias de Importância Médica. Accessed Dec. 30, 2022. https://www.gov.br/anvisa/pt-br/centraisdeconteudo/publicacoes/servicosdesaude/publicacoes/modulo-6-deteccao-e-identificacao-de-bacterias-de-importancia-medica/view.

Brito, M. A. V. P., J. R. F. Brito, M. T. Ribeiro, and V. M. O. Veiga. 1999. Dairy herds pattern of intramammary infection: evaluation of all mammary quarters of lactating cows. Arq. Bras. Med. Vet. Zootec. 51:129-135. https://doi.org/10.1590/S010209351999000200001.

Chakkera, H. A., J. D. Schold, and B. Kaplan. 2016. P Value: significance is not all black and white. Transplantation 100:1607-1609. https://doi.org/10.1097/tp.0000000000001331.

Churakov, M., J. Katholm, S. Rogers, R. R. Kao, and R. N. Zadoks. 2021. Assessing potential routes of *Streptococcus agalactiae* transmission between dairy herds using national surveillance, animal movement data and molecular typing. Prev. Vet. Med. 197:105501-105511. https://doi.org/10.1016/j.prevetmed.2021.105501.

Condas, L. A. Z., J. De Buck, D. B. Nobrega, D. A. Carson, J. Roy, G. P. Keefe, T. J. DeVries, J. R. Middleton, S. Dufour, and H. W. Barkema. 2017. Distribution of non-*aureus* staphylococci species in udder quarters with low and high somatic cell count, and clinical mastitis. J. Dairy Sci. 100:1-15. https://doi.org/10.3168/jds.2016-12479.

Côte-Gravel, J., and F. Malouin. 2019. Symposium review: Features of *Staphylococcus aureus* mastitis pathogenesis that guide vaccine development strategies. J. Dairy Sci. 102:4727-4740. https://doi.org.10.3168/jds.2018-15272.

Crestani, C., T. L. Forde, S. J. Lycett, M. A. Holmes, C. Fasth, K. Persson-Waller, and R. N. Zudoks. 2021. The fall and rise of group b *Streptococcus* in dairy cattle: reintroduction due to human-to-cattle host jumps. Microb. Genom. 7:648-658. https://doi.org/10.1099/mgen.0.000648.

Dalanezi, F. M., S. F. Joaquim, F. F. Guimarães, S. T. Guerra, B. C. Lopes, E. M. S. Schmidt, R. L. A. Cerri, and H. Langoni. 2020. Influence of pathogens causing clinical mastitis on reproductive variables of dairy cows. J. Dairy Sci. 103:3648-3655. https://doi.org/10.3168/jds.2019-16841.

De Buck, J., V. Ha, S. Naushad, D. B. Nobrega, C. Luby, J. R. Middleton, S. De Vliegher, and H. W. Barkema. 2021. Non-aureus staphylococci and bovine udder health: current understanding and knowledge gaps. Front. Vet. Sci. 8:1-16. https://doi.org/10.3389/fvets.2021.658031.

De Visscher, A., S. Piepers, F. Haesebrouck, and S. De Vliegher. 2016. Intramammary infection with coagulase-negative staphylococci at parturition: Species-specific prevalence, risk factors, and effect on udder health. J. Dairy Sci. 99:6457-6469. http://dx.doi.org/10.3168/jds.2015-10458.

DeGraves, F. J., and J. Fetrow. 1993. Economics of mastitis and mastitis control. Vet. Clin. North Am. Food Anim. Pract. 9:421–434. https://doi.org/10.1016/s0749-0720(15)30611-3.

Dinsmore, R. P., P. B. English, R. N. Gonzalez, P. M. Sears, and H. F. Schulte. 1991. Evaluation of methods for the diagnosis of Streptococcus agalactiae intramammary infections in Dairy Cattle. J. Dairy Sci. 74:1521-1526. https://doi.org/10.3168/jds.s0022-0302(91)78312-4.

Dohoo, I. R., J. Smith, S. Andersen, D. F. Kelton, S. Godden, and Mastitis Research Workers' Conference. 2011. Diagnosing intramammary infections: evaluation of definitions based on a single milk sample. J. Dairy Sci. 94:250-261. https://doi.org/10.3168/jds.2010-3559.

Dyson, R., N. Charman, A. Hodge, S. M. Rowe, and L. F. Taylor. 2022. A survey of mastitis pathogens including antimicrobial susceptibility in southeastern Australian dairy herds. J. Dairy Sci. 105:1504-1518. https://doi.org/10.3168/jds.2021-20955.

Ferguson, J. D., G. Azzaro, M. Gambina, and G. Licitra. 2007. Prevalence of mastitis pathogens in Ragusa, Sicily, from 2000 to 2006. J. Dairy Sci. 90:5798-5813. https://doi.org/10.3168/jds.2006-903.

Foret, C. J., C. Corbellini, S. Young, and P. Janowicz. 2005. Efficacy of two iodine teat dips based on reduction of naturally occurring new intramammary infections. J. Dairy Sci. 88:426-432. https://doi.org/10.3168/jds.S0022-0302(05)72704-1.

Fox, L. K., and J. M. Gay. 1993. Contagious mastitis. Vet. Clin. North Am. Food Anim. Pract. 9:475-487. https://doi.org/10.1016/s0749-0720(15)30615-0.

Gao J., H. W. Barkema, L. Zhang, G. Liu, Z. Deng, L. Cai, R. Shan, S. Zhang, J. Zou, J. P. Kastelic, and B. Han. 2017. Incidence of clinical mastitis and distribution of pathogens on large Chinese dairy farms. J. Dairy Sci. 100:4797-4806. https://doi.org/10.3168/jds.2016-12334.

Garrity, G. M., and J. G. Holt. 2001a. The road map to the manual. Pages 119-166 in Bergey's Manual of Systematic Bacteriology. D. R. Boone, R. W. Castenholz, ed. Springer Verlag, New York, USA.

Gonçalves, J. L., T. Tomazi, J. R. Barreiro, D. C. Beuron, M. A. Arcari, S. H. Lee, C. M. M. R. Martins, J. P. A. Júnior, and M. V. Santos. 2016. Effects of bovine subclinical mastitis caused by *Corynebacterium* spp on somatic cell count, milk yield and composition by comparing contralateral quarters. Vet. J. 209:87-92. https://doi.org/10.1016/j.tvjl.2015.08.009.

Hertl, J. A., Y. H. Schukken, F. L. Welcome, L. W. Tauer, and Y. T. Gröhn. 2014. Pathogen-specific effects on milk yield in repeated clinical mastitis episodes in Holstein dairy cows. J. Dairy Sci. 97:1465-1480. https://doi.org/10.3168/jds.2013-7266.

Hogan, J. S., R. N. González, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith. 2005. *Streptococci* and related genera in Laboratory handbook on bovine mastitis. 3rd edition. ed. National Mastitis Council, Inc., New Prague, MN, USA.

Hogan, J. S., R. N. González, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith. 2005. *Staphylococci* in Laboratory handbook on bovine mastitis. 3rd edition. ed. National Mastitis Council, Inc., New Prague, MN, USA.

Hogan, J. S., R. N. González, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith. 2005. Gram-negative bacteria in Laboratory handbook on bovine mastitis. 3rd edition. ed. National Mastitis Council, Inc., New Prague, MN, USA.

Hogan, J. S., R. N. González, R. J. Harmon, S. C. Nickerson, S. P. Oliver, J. W. Pankey, and K. L. Smith. 2005. Miscellaneous organisms in Laboratory handbook on bovine mastitis. 3rd edition. ed. National Mastitis Council, Inc., New Prague, MN, USA.

Holt, J. G, N. R. Krieg, P. H. A. Sneath, J. T. Staley, and S. T. Williams. 1994. Gram-negative aerobic/microaerophilic rods and cocci in Bergey´s Manual of Determinative Bacteriology. 9th edition. William R. Hensyl, ed. Lippincott Williams & Wilkins, Baltimore, USA.

Holt, J. G, Krieg N. R., P. H. A. Sneath, J. T. Staley, and S. T. Williams. 1994. Facultatively anaerobic gram-negative rods in Bergey´s Manual of Determinative Bacteriology. 9th edition. William R. Hensyl, ed. Lippincott Williams & Wilkins, Baltimore, USA.

Jenkins, S. N., E. Okello, P. V. Rossitto, T. W. Lehenbauer, J. Champagne, M. C. T. Penedo, A. G. Arruda, S. Godden, P. Rapnicki, P. J. Gorden, L. L. Timms, and S. S. Aly. 2019. Molecular epidemiology of coagulase-negative *Staphylococcus* species isolated at different lactation stages from dairy cattle in the United States. PeerJ. 6749:1-25. https://pubmed.ncbi.nlm.nih.gov/31119068/.

Jørgensen, H. J., A. B. Nordstoga, S. Svilland, R. N. Zadoks, L. Sølverød, B. Kvitle, and T. Mørk. 2016. *Streptococcus agalactiae* in the environment of bovine dairy herds - rewriting the textbooks? Vet. Microbiol. 184:64-72. https://doi.org/10.1016/j.vetmic.2015.12.014.

Koivula, M., A. Pitkälä, S. Pyörälä, and E. A. Mäntysaari. 2007. Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland. Acta Agric. Scand. Sec. A. Anim Sci. 57:89-96. https://doi.org/10.1080/09064700701488941.

Langoni, H., D. S. Penachio, J. C. C. Citadella, F. Laurino, P. Y. Faccioli-Martins, S. B. Lucheis, B. D. Menozzi, and A. V. Silva. 2011. Aspectos microbiológicos e de qualidade do leite bovino. Pesq. Vet. Bras. 31:1059-1065. https://doi.org/10.1590/S010073_6X2011001200004.

Makovec, J. A., and P. L. Ruegg. 2003. Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. J. Dairy Sci. 86:3466-3472. https://doi.org/10.3168/jds.S0022-0302(03)73951-4.

NAHMS. 2021. Dairy 2014. Trends in Dairy Cattle Health and Management Practices in the United States, 1991-2014. Accessed Nov. 25, 2022. https://www.aphis.usda.gov/animalhealth/nahms/dairy/downloads/dairy-trends-hlth-ngmnt-1991-2014.pdf.

Oliveira, L., C. Hulland, and Ruegg, P. L. 2013. Characterization of clinical mastitis occurring in cows on 50 large dairy herds in Wisconsin. J. Dairy Sci. 96:7538-7549. https://doi.org/10.3168/jds.2012-6078.

Østerås, O., L. Sølverød, and O. Reksen. 2006. Milk culture results in a large Norwegian survey-effects of season, parity, days in milk, resistance, and clustering. J. Dairy Sci. 89:1010-1023. https://doi.org/10.3168/jds.S0022-0302(06)72167-1.

Piepers, S., L. De Meulemeester, A. de Kruif, G. Opsomer, H. W. Barkema, and S. De Vliegher. 2007. Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. J. Dairy Res. 74:478-483. https://doi.org/10.1017/S0022_029907002841.

Piessens, V., E. V. Coillie, B. Verbist, K. Supré, G. Braem, A. V. Nuffel, L. De Vuyst, M. Heyndrickx, and S. De Vliegher. 2011. Distribution of coagulase-negative *Staphylococcus* species from milk and environment of dairy cows differs between herds. J. Dairy Sci. 94:2933-2944. https://pubmed.ncbi.nlm.nih.gov/21605763/.

Pitkälä, A., M. Haveri, S. Pyörälä, V. Myllys, and T. Honkanen-Buzalski. 2004. Bovine mastitis in Finland 2001 - prevalence, distribution of bacteria, and antimicrobial resistance. J. Dairy Sci. 87:2433–2441. https://doi.org/10.3168/jds.S0022-0302(04)73366-4.

Reyher, K. K., and I. R. Dohoo. 2011. Diagnosing intramammary infections: evaluation of composite milk samples to detect intramammary infections. J. Dairy Sci. 94:3387-3396. https://doi.org/103168/jds.2010-3907.

Rossitto P. V., L. Ruiz, Y. Kikuchi, K. Glenn, K. Luiz, J. L. Watts, and J. S. Cullor. 2002. Antibiotic susceptibility patterns for environmental streptococci isolated from bovine mastitis in central California dairies. J. Dairy Sci. 85:132-138. https://doi.org/10.3168/jds.S0022-0302(02)74061-7.

Ruegg, P. L. 2017. A 100-Year Review: Mastitis detection, management, and prevention. J. Dairy Sci. 100:10381–10397. https://doi.org/10.3168/jds.2017-13023.

Santos, J. E. P., R. L. A. Cerri, M. A. Ballou, G. E. Higginbotham, and J. H. Kirk. 2004. Effect of timing of first clinical mastitis occurrence on lactational and reproductive performance of Holstein dairy cows. Anim. Reprod. Sci. 80:31-45. https://doi.org/10.1016/s0378-4320(03)00133-7.

Sargeant, J. M., and A. M. O'Connor. 2014. Issues of reporting in observational studies in veterinary medicine. Prev. Vet. Med. 113:323-330. https://doi.org/10.106/j.prevetmed.2013.09.004.

Schrick, F. N., M. E. Hockett, A. M. Saxton, M. J. Lewis, H. H. Dowlen, and S. P. Oliver. 2001. Influence of subclinical mastitis during early lactation on reproductive parameters. J. Dairy Sci. 84:1407-1412. https://doi.org/10.3168/jds.S0022-0302(01)70172-5.

Schukken, Y. H., F. J. Grommers, J. A. Smit, D. Vandegeer, and A. Brand. 1989. Effect of freezing on bacteriological culturing of mastitis milk samples. J. Dairy Sci. 72:1900-1906. https:// doi.org/10.3168/jds.s0022-0302(89)79309-7.

Schukken, Y. H., R. N. González, L. L. Tikofsky, H. F. Schulte, C. G. Santisteban, F. L. Welcome, G. J. Bennett, M. J. Zurakowski, and R. N. Zadocks. 2009. CNS mastitis: Nothing to worry about? Vet. Microb. 134:9-14. http://dx.doi.org/10.1016/j.vetmic. 2008.09.014.

Smith, K. L., D. A. Todhunter, and P. S. Schoenberger. 1985. Environmental mastitis: cause, prevalence, prevention. J. Dairy Sci. 68:1531-1553. https://doi.org/10.3168/jds.S00220302(85)80993-0.

Tenhagen, B. A., G. Köster, J. Wallmann, and W. Heuwieser. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. J. Dairy Sci. 89:2542-2551. https://doi.org/10.3168/jds.s0022-0302(06)72330-x.

Tomazi, T., G. C. Ferreira, A. M. Orsi, J. L. Gonçalves, P. A. Ospina, D. V. Nydam, P. Moroni, and M. V. dos Santos. 2018. Association of herd-level risk factors and incidence rate of clinical mastitis in 20 Brazilian dairy herds. Prev. Vet. Med. 161:9-18. https://doi.org/10.1016/j.prevetmed.2018.10.007.

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Watts, J. L. 1988. Etiological agents of bovine mastitis. Vet. Microbiol. 16:41-66. https://doi.org/10.1016/0378-1135(88)90126-5.

Zadocks, R. N., B. E. Gillespie, H. W. Barkema, O. C. Sampimon, S. P. Oliver, and Y. H. Schukken. 2003. Clinical, epidemiological and molecular characteristics of *Streptococcus uberis* infections in dairy herds. Epidemiol. Infect. 130:335-349. https://doi.org/10.1017/s0950268802008221.

**Table 1.** Proportion of the 3,793 farms with at least one positive microbiological result of the most prevalent microorganisms during the period of 2012 to July 2016, and from August 2016 to 2020

|  | 2012-2016 | 2016-2020 | Difference (p.p.)[1] |
|---|---|---|---|
| Farms,[2] n | 1,858 | 2,399 |  |
| Culture results[3,4] |  |  |  |
|   Contagious, % (n) | 90.2 (1,676) | 85.5 (2,051) | -4.70 |
|   Environmental, % (n) | 88.9 (1,651) | 91.1 (2,186) | 2.20 |
| Specified pathogens[5] |  |  |  |
|   NAS, % (n) | 81.3 (1,511) | 84.7 (2,032) | 3.40 |
|   *S. chromogenes,* % (n) | 58.3 (1,083) | 68.7 (1,649) | 10.4 |
|   *S. aureus*, % (n) | 76.8 (1,427) | 67.7 (1,625) | -9.10 |
|   *S. agalactiae*, % (n) | 66.8 (1,241) | 54.2 (1,301) | -12.6 |
|   *C. bovis*, % (n) | 64.4 (1,197) | 62.3 (1,495) | -2.10 |
|   *Klebsiella* spp, % (n) | 60.4 (1,122) | 49.4 (1,184) | -11.0 |
|   *S. uberis*, % (n) | 49.6 (922) | 66.0 (1,583) | 16.4 |
|   *E. coli*, %, (n) | 46.0 (855) | 33.8 (811) | -12.2 |
|   *S. dysgalactiae*, % (n) | 33,1 (615) | 37,6 (903) | 4,50 |
|   *Pseudomonas* spp, % (n) | 40.3 (749) | 38.0 (911) | -2.32 |

[1] p.p. = percentage points.

[2] Number of farms that submitted samples in the respective study period.

[3] Milk samples submitted to a commercial diagnostic laboratory and cultured according to NMC (2005) guidelines. n = represents the number of positive samples for the respective group or specified pathogen.

[4] Contagious pathogens = *Staphylococcus aureus, Streptococcus agalactiae* and *Corynebacterium bovis*. Environmental pathogens = coliforms, environmental streptococci, environmental staphylococci, enterococci, lactococci, and *Pseudomonas* spp.

[5] Proportion and number of farms that presented at least 1 positive result for the most prevalent groups of microorganisms or individual microorganisms isolated from milk. Exclude contaminated results. NAS = non-aureus staphylococci.

**Table 2.** The most prevalent microorganisms isolated from milk samples submitted by 3,793 dairy farms in Brazil and by the 12 farms that submitted samples all 9 years, and the distribution of samples according to type of mastitis

| | 3,793 farms | | | 12 farms | | |
|---|---|---|---|---|---|---|
| | All samples | CM[1] | SCM[1] | All samples | CM[1] | SCM[1] |
| Milk samples, n[2] | 679,706 | 15,407 | 2,535 | 193,914 | 9,074 | 1,546 |
| Specified pathogens[3] | | | | | | |
| NAS, % (n) | 23.6 | 7.06 | 27.1 | 26.4 | 6.51 | 31.6 |
| | (160,239) | (1,088) | (688) | (51,271) | (591) | (489) |
| *S. chromogenes*, % (n) | 10.3 | 2.88 | 15.1 | 11.5 | 2.84 | 19.3 |
| | (70,213) | (444) | (382) | (22,343) | (258) | (299) |
| *S. aureus*, % (n) | 7.62 | 1.23 | 2.05 | 1.61 | 0.87 | 1.55 |
| | (51,788) | (190) | (52) | (3,128) | (79) | (24) |
| *S. agalactiae*, % (n) | 7.08 | 17.7 | 1.89 | 4.46 | 21.3 | 1.23 |
| | (48,115) | (2,725) | (48) | (8,647) | (1,931) | (19) |
| *C. bovis*, % (n) | 6.91 | 1.98 | 6.00 | 3.44 | 1.32 | 6.66 |
| | (46,980) | (305) | (152) | (6,671) | (120) | (103) |
| *Klebsiella* spp, % (n) | 5.21 | 8.22 | 4.18 | 6.28 | 7.85 | 3.23 |
| | (35,433) | (1,267) | (106) | (12,174) | (712) | (50) |
| *S. uberis*, % (n) | 4.84 | 12.2 | 7.26 | 4.18 | 12.5 | 6.60 |
| | (32,895) | (1,885) | (184) | (8,098) | (1,132) | (102) |
| *E. coli*, % (n) | 2.79 | 6.21 | 1.62 | 3.01 | 5.55 | 1.03 |
| | (18,977) | (957) | (41) | (5,844) | (504) | (16) |
| *S. dysgalactiae*, % (n) | 1.40 | 1.76 | 2.17 | 0.96 | 1.77 | 2.46 |
| | (9,538) | (271) | (55) | (1,862) | (161) | (38) |
| *Pseudomonas* spp, % (n) | 2.98 | 2.73 | 2.37 | 3.55 | 3.10 | 2.20 |
| | (20,283) | (420) | (60) | (6,889) | (281) | (34) |

[1] CM = clinical mastitis; SCM = subclinical mastitis.

[2] Milk samples submitted to a commercial diagnostic laboratory and cultured according to NMC (2005) guidelines. For all samples, CM and SCM, contaminated microbiological results were excluded. n = represents the number of samples analyzed or the number of positive samples for the respective group or specified pathogen.

[3] The most prevalent groups of microorganisms or individual microorganisms isolated from milk with growth representing 72.7% (n = 494,461) of the milk samples submitted by 3,793 farms and

65.5% (n = 126,927) of milk samples submitted by the 12 farms. NAS = non-aureus staphylococci.

**Table 3**. Association between the type of mastitis and the pathogen isolated from milk samples submitted by 3,793 dairy farms in Brazil and by the 12 farms that submitted samples all 9 years (LSM ± SEM)[1]

| | 3,793 farms | | | | 12 farms | | | |
|---|---|---|---|---|---|---|---|---|
| | Clinical | Subclinical | AOR (95% CI)[2] | *P*-value[3] | Clinical | Subclinical | AOR (95% CI) | *P*-value |
| Milk samples, n[4] | 15,407 | 2,535 | --- | --- | 9,074 | 1,546 | --- | --- |
| Specified pathogens[5] | | | | | | | | |
| NAS, % (n) | 9.34 ± 1.01 (1,088) | 24.5 ± 2.47 (688) | 3.15 (2.68-3.70) | < 0.001 | 6.92 ± 0.95 (591) | 29.0 ± 3.1 (489) | 5.49 (4.56-6.61) | < 0.001 |
| *S. chromogenes*, % (n) | 3.14 ± 0.54 (444) | 10.3 ± 1.78 (382) | 3.54 (2.80-4.48) | < 0.001 | 2.95 ± 0.53 (258) | 15.8 ± 2.48 (299) | 6.16 (4.74-8.00) | < 0.001 |
| *S. aureus*, % (n) | 3.82 ± 0.74 (190) | 4.30 ± 1.06 (52) | 1.13 (0.75-1.7) | 0.56 | 2.74 ± 1.25 (79) | 2.88 ± 1.40 (24) | 1.05 (0.55-2.02) | 0.88 |
| *S. agalactiae*, % (n) | 17.4 ± 0.46 (2725) | 1.98 ± 0.29 (48) | 0.10 (0.07-0.13) | < 0.001 | 16.1 ± 1.36 (1931) | 1.34 ± 0.29 (19) | 0.07 (0.05-0.11) | < 0.001 |
| *C. bovis*, % (n) | 2.21 ± 0.31 (305) | 6.28 ± 0.95 (152) | 2.96 (2.34-3.75) | < 0.001 | 2.96 ± 1.24 (120) | 7.77 ± 3.14 (103) | 2.77 (1.78-4.32) | < 0.001 |
| *Klebsiella* spp, % (n) | 7.47 ± 1.13 (1,267) | 3.96 ± 0.74 (106) | 0.51 (0.40-0.65) | < 0.001 | 10.2 ± 2.24 (712) | 5.77 ± 1.48 (50) | 0.54 (0.37-0.79) | < 0.001 |
| *S. uberis*, % (n) | 7.43 ± 1.29 (1,885) | 3.85 ± 0.76 (184) | 0.50 (0.41-0.61) | < 0.001 | 8.84 ± 1.79 (1,132) | 5.54 ± 1.25 (102) | 0.60 (0.45-0.80) | < 0.001 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *E. coli*, % (n) | 7.96 ± 1.26 (957) | 1.05 ± 0.27 (41) | 0.12 (0.08-0.19) | < 0.001 | 11.2 ± 1.63 (504) | 1.08 ± 0.32 (16) | 0.09 (0.05-0.18) | < 0.001 |
| *S. dysgalactiae*, % (n) | 2.11 ± 0.28 (271) | 2.48 ± 0.46 (55) | 1.18 (0.85-1.64) | 0.32 | 2.32 ± 0.44 (161) | 2.60 ± 0.60 (38) | 1.13 (0.71-1.79) | 0.61 |
| *Pseudomonas* spp, % (n) | 2.31 ± 0.51 (420) | 2.10 ± 0.56 (60) | 0.91 (0.62-1.34) | 0.63 | 3.49 ± 1.30 (281) | 2.51 ± 1.03 (34) | 0.71 (0.40-1.25) | 0.23 |

[1] Milk samples submitted to a commercial diagnostic laboratory with identification of the type of mastitis and cultured according to NMC (2005) guidelines.

[2] AOR = adjusted odds ratio; CI = confidence interval. Subclinical mastitis was the reference for comparison.

[3] Within a row and the specific group of farms, the *P*-value refers to the comparison between clinical and subclinical mastitis.

[4] Number of milk samples that had either no growth or growth in the microbiological result. They exclude all samples with contaminated results.

[5] The most prevalent groups of microorganisms or individual microorganisms isolated from milk with growth representing 2.64% (n = 17,942) of the milk samples submitted by 3,793 farms and 5.48% (n = 10,620) of milk samples submitted by the 12 farms with identification of the type of mastitis. NAS = non-aureus staphylococci

**Table 4**. Results of microbiological culture of milk samples according to the year of submission from 3,793 dairy farms in Brazil (LSM ± SEM)[1]

| | Year | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 |
| Milk samples analyzed, n | 68,441 | 55,146 | 75,355 | 104,368 | 131,062 | 89,421 | 70,048 | 63,709 | 59,618 |
| Culture results[2] | | | | | | | | | |
| No growth,[*] % (n) | 30.2 ± 0.41 | 29.4 ± 0.42 | 37.3 ± 0.44 | 33.8 ± 0.53 | 34.9 ± 0.52 | 32.1 ± 0.51 | 24.2 ± 0.30 | 21.6 ± 0.36 | 27.0 ± 0.46 |
| | (25,027) | (18,507) | (32,172) | (47,320) | (54,685) | (35,091) | (17,657) | (17,504) | (18,285) |
| Contaminated,[*] % (n) | 2.25 ± 0.13 | 3.05 ± 0.16 | 4.39 ± 0.22 | 7.17 ± 0.22 | 2.67 ± 0.15 | 3.49 ± 0.14 | 4.11 ± 0.14 | 7.05 ± 0.20 | 7.03 ± 0.30 |
| | (1,552) | (2,192) | (3,565) | (6,007) | (5,384) | (3,805) | (3,206) | (5,461) | (6,290) |
| Growth,* % (n) | 66.1 ± 0.42 | 66.5 ± 0.44 | 57.4 ± 0.46 | 57.4 ± 0.5 | 62.3 ± 0.55 | 64.1 ± 0.42 | 71.5 ± 0.32 | 70.7 ± 0.39 | 64.4 ± 0.57 |
| | (41,862) | (34,447) | (39,618) | (51,041) | (70,993) | (50,525) | (49,185) | (40,744) | (35,043) |
| Contagious, * % (n) | 28.7 ± 0.41 | 30.5 ± 0.43 | 26.8 ± 0.39 | 27.1 ± 0.46 | 25.9 ± 0.44 | 22.6 ± 0.34 | 24.3 ± 0.35 | 27.2 ± 0.40 | 26.6 ± 0.58 |
| | (17,854) | (13,629) | (15,620) | (17,835) | (18,488) | (13,518) | (15,977) | (13,715) | (13,448) |
| Environmental,* % (n) | 26.3 ± 0.41 | 27.7 ± 0.43 | 22.0 ± 0.41 | 23.5 ± 0.46 | 24.8 ± 0.48 | 24.2 ± 0.37 | 36.1 ± 0.35 | 30.0 ± 0.40 | 26.4 ± 0.56 |
| | (16,854) | (15,426) | (16,201) | (22,282) | (32,884) | (20,624) | (23,990) | (18,796) | (13,976) |
| Specified pathogens[3] | | | | | | | | | |
| NAS,[*] % (n) | 19.0 ± 0.35 | 20.0 ± 0.40 | 18.4 ± 0.35 | 17.1 ± 0.40 | 20.8 ± 0.48 | 25.9 ± 0.38 | 28.7 ± 033 | 30.2 ± 0.41 | 30.0 ± 0.60 |
| | (11,996) | (10,566) | (12,755) | (17,430) | (30,905) | (22,949) | (19,126) | (18,509) | (16,003) |
| *S. chromogenes*,[*] % (n) | 3.53 ± 0.16 | 5.23 ± 0.21 | 5.74 ± 0.21 | 6.25 ± 0.24 | 5.50 ± 0.28 | 7.74 ± 0.24 | 16.0 ± 0.28 | 15.5 ± 0.34 | 18.1 ± 0.53 |
| | (2,384) | (2,669) | (4,042) | (6,360) | (12,290) | (10,364) | (11,977) | (10,139) | (9,988) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *S. aureus*,[*] % (n) | 10.9 ± 0.28 | 9.45 ± 0.26 | 9.54 ± 0.24 | 12.8 ± 0.33 | 8.77 ± 0.26 | 7.04 ± 0.23 | 9.20 ± 0.23 | 11.6 ± 0.29 | 10.3 ± 0.42 |
| | (6,092) | (4,543) | (5,613) | (8,150) | (6,253) | (4,693) | (5,673) | (5,777) | (4,994) |
| *S. agalactiae*,[*] % (n) | 7.19 ± 0.23 | 9.40 ± 0.28 | 7.57 ± 0.23 | 8.55 ± 0.27 | 8.75 ± 0.25 | 6.20 ± 0.20 | 6.14 ± 0.22 | 6.40 ± 0.23 | 3.54 ± 0.70 |
| | (5,935) | (4,287) | (5,277) | (6,346) | (7,090) | (4,944) | (5,622) | (4,590) | (4,024) |
| *C. bovis*,[*] % (n) | 10.2 ± 0.27 | 11.7 ± 0.30 | 8.70 ± 0.25 | 4.74 ± 0.28 | 8.03 ± 0.21 | 7.60 ± 0.22 | 8.71 ± 0.22 | 7.10 ± 0.20 | 10.1 ± 0.37 |
| | (6,713) | (5,395) | (5,504) | (4,242) | (5,879) | (4,362) | (5,507) | (4,150) | (5,228) |
| *Klebsiella* spp,[*] % (n) | 5.17 ± 0.27 | 5.88 ± 0.30 | 6.40 ± 0.25 | 5.25 ± 0.28 | 4.71 ± 0.21 | 4.73 ± 0.22 | 4.76 ± 0.22 | 2.57 ± 0.20 | 1.62 ± 0.37 |
| | (3,668) | (3,579) | (4,735) | (5,177) | (6,760) | (4,657) | (3,673) | (2,022) | (1,162) |
| *S. uberis*,[*] % (n) | 0.61 ± 0.13 | 1.84 ± 0.17 | 1.89 ± 0.14 | 5.07 ± 0.22 | 6.05 ± 0.27 | 8.38 ± 0.24 | 8.33 ± 0.24 | 4.48 ± 0.18 | 4.70 ± 0.38 |
| | (644) | (1,472) | (1,842) | (4,777) | (6,955) | (5,850) | (5,437) | (2,941) | (2,977) |
| *E. coli*,[*] % (n) | 5.08 ± 0.22 | 4.43 ± 0.22 | 2.34 ± 0.15 | 1.97 ± 0.16 | 1.75 ± 0.13 | 1.98 ± 0.12 | 2.36 ± 0.12 | 1.10 ± 0.12 | 1.05 ± 0.13 |
| | (3,530) | (2,836) | (2,019) | (2,290) | (2,514) | (1,807) | (2,039) | (1,171) | (771) |
| *S. dysgalactiae*,[*] % (n) | 1.11 ± 0.11 | 2.19 ± 0.17 | 1.22 ± 0.13 | 0.61 ± 0.06 | 0.81 ± 0.10 | 0.51 ± 0.05 | 2.26 ± 0.11 | 1.60 ± 0.11 | 1.48 ± 0.18 |
| | (972) | (1,288) | (1,107) | (659) | (1,224) | (522) | (1,520) | (1,137) | (1,109) |
| *Pseudomonas* spp,[*] % (n) | 2.02 ± 0.12 | 3.56 ± 0.17 | 2.96 ± 0.15 | 3.79 ± 0.23 | 2.39 ± 0.17 | 1.83 ± 0.11 | 2.58 ± 0.11 | 2.66 ± 0.15 | 2.12 ± 0.18 |
| | (1,406) | (2,183) | (2,190) | (3,655) | (3,657) | (1,720) | (1,961) | (2,289) | (1,222) |

[*] Effect of the association with year ($P < 0.001$).

[1] Milk samples submitted to a commercial diagnostic laboratory and cultured according to NMC (2005) guidelines.

[2] Contaminated = growth of 1or more colonies from 4 or more different microorganisms in culture media. Growth = 3 or more colonies for up to 3 species, except for *Staphylococcus aureus* and *Streptococcus agalactiae* which 1 colony was defined as a growth result. Contagious pathogens = *Staphylococcus aureus, Streptococcus agalactiae* and *Corynebacterium bovis*. Environmental pathogens = coliforms, environmental streptococci, environmental

staphylococci, enterococci, lactococci and *Pseudomonas* spp. n = represents the number of samples analyzed or the number of positive samples for the respective group or specified pathogen.

[3] Microbiological results of the most prevalent microorganisms isolated from culture media, excluding contaminated results, representing 72.7% (n = 494,461) of 679,706 milk samples submitted to a commercial diagnostic laboratory from 2012 to 2020. NAS = non-aureus staphylococci.

**Table 5**. Results of microbiological culture of milk samples according to the year of submission from the 12 selected farms that submitted samples all 9 years (LSM ± SEM)[1]

| | Year | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2012 | 2013 | 2014 | 2015 | 2016 | 2017 | 2018 | 2019 | 2020 |
| Milk samples analyzed, n | 13,448 | 12,089 | 17,664 | 38,482 | 58,138 | 32,873 | 17,687 | 10,735 | 3,345 |
| Culture results[2] | | | | | | | | | |
| No growth,* % (n) | 31.9 ± 2.76 | 29.2 ± 2.57 | 44.5 ± 3.03 | 41.1 ± 2.98 | 46.2 ± 3.06 | 39.0 ± 2.93 | 28.3 ± 2.52 | 32.7 ± 2.72 | 31.7 ± 2.93 |
| | (6,099) | (4,318) | (8,303) | (20,115) | (27,261) | (13,212) | (4,271) | (3,770) | (1,076) |
| Contaminated,* % (n) | 2.17 ± 0.72 | 3.07 ± 0.96 | 2.74 ± 0.84 | 3.21 ± 0.98 | 2.20 ± 0.68 | 4.32 ± 1.31 | 3.03 ± 0.94 | 5.64 ± 1.68 | 5.38 ± 1.68 |
| | (413) | (869) | (584) | (1,918) | (2,791) | (1,857) | (986) | (676) | (453) |
| Growth,* % (n) | 64.5 ± 2.46 | 61.1 ± 2.47 | 51.8 ± 2.57 | 53.3 ± 2.57 | 50.1 ± 2.58 | 54.7 ± 2.56 | 66.8 ± 2.32 | 60.3 ± 2.48 | 56.7 ± 2.84 |
| | (6,936) | (6,902) | (8,777) | (16,449) | (28,086) | (17,804) | (12,430) | (6,289) | (1,816) |
| Contagious,* % (n) | 15.1 ± 3.16 | 20.0 ± 3.93 | 15.8 ± 3.26 | 11.4 ± 2.47 | 6.42 ± 1.48 | 6.04 ± 1.40 | 5.80 ± 1.37 | 4.12 ± 0.99 | 8.02 ± 1.99 |
| | (1,467) | (1,781) | (2,503) | (3,714) | (3,229) | (2,275) | (2,268) | (651) | (259) |
| Environmental,* % (n) | 38.2 ± 3.70 | 32.9 ± 3.70 | 26.0 ± 2.97 | 29.3 ± 3.20 | 26.8 ± 3.03 | 26.6 ± 3.02 | 41.1 ± 3.76 | 33.3 ± 3.44 | 25.1 ± 3.14 |
| | (3,846) | (3,920) | (4,395) | (8,322) | (13,997) | (7,017) | (7,055) | (3,034) | (805) |
| Specified pathogens[3] | | | | | | | | | |
| NAS,* % (n) | 19.9 ± 1.39 | 21.1 ± 1.37 | 16.5 ± 1.12 | 20.1 ± 1.29 | 23.4 ± 1.45 | 30.1 ± 1.70 | 35.9 ± 1.88 | 42.8 ± 1.97 | 46.8 ± 2.44 |
| | (2,476) | (2,197) | (3,016) | (6,774) | (15,216) | (10,669) | (5,419) | (4,198) | (1,306) |
| *S. chromogenes*,* % (n) | 3.12 ± 0.33 | 5.10 ± 0.37 | 5.64 ± 0.36 | 7.10 ± 0.43 | 10.2 ± 0.58 | 15.1 ± 0.80 | 20.7 ± 1.03 | 24.3 ± 1.11 | 28.2 ± 1.81 |
| | (461) | (587) | (998) | (2,436) | (6,387) | (5,033) | (3,203) | (2,447) | (791) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *S. aureus*,[*] % (n) | 3.57 ± 1.18 | 3.91 ± 1.26 | 2.33 ± 0.76 | 3.73 ± 1.20 | 1.34 ± 0.45 | 0.70 ± 0.24 | 1.42 ± 0.48 | 1.16 ± 0.40 | 2.47 ± 0.93 |
| | (458) | (450) | (445) | (840) | (408) | (127) | (203) | (124) | (73) |
| *S. agalactiae*,[*] % (n) | 1.84 ± 0.88 | 5.66 ± 2.58 | 2.80 ± 1.31 | 2.08 ± 0.98 | 0.90 ± 0.43 | 0.91 ± 0.44 | 0.59 ± 0.32 | 0.30 ± 0.15 | 0.21 ± 0.16 |
| | (230) | (732) | (946) | (1,735) | (1,504) | (1,479) | (1,717) | (292) | (12) |
| *C. bovis*,[*] % (n) | 7.68 ± 1.73 | 5.99 ± 1.37 | 6.91 ± 1.55 | 4.85 ± 0.94 | 4.05 ± 0.94 | 2.43 ± 0.58 | 1.73 ± 0.44 | 2.06 ± 0.50 | 5.92 ± 1.57 |
| | (802) | (652) | (1,172) | (1,237) | (1,349) | (679) | (357) | (245) | (178) |
| *Klebsiella* spp,[*] % (n) | 11.5 ± 1.56 | 9.70 ± 1.32 | 9.44 ± 1.27 | 6.59 ± 0.92 | 6.03 ± 0.85 | 6.75 ± 0.95 | 5.98 ± 0.87 | 2.71 ± 0.43 | 0.78 ± 0.27 |
| | (986) | (1,134) | (1,573) | (1,897) | (3,460) | (1,630) | (1,178) | (274) | (42) |
| *S. uberis*,[*] % (n) | 1.22 ± 0.23 | 2.43 ± 0.40 | 2.16 ± 0.34 | 3.71 ± 0.56 | 3.73 ± 0.57 | 5.31 ± 0.78 | 5.93 ± 0.88 | 2.98 ± 0.48 | 1.40 ± 0.42 |
| | (145) | (356) | (417) | (1,556) | (2,387) | (1,554) | (1,241) | (375) | (67) |
| *E. coli*,[*] % (n) | 9.06 ± 1.15 | 7.20 ± 0.90 | 3.19 ± 0.42 | 2.00 ± 0.28 | 2.22 ± 0.31 | 3.41 ± 0.45 | 2.29 ± 0.36 | 1.58 ± 0.24 | 1.12 ± 0.34 |
| | (865) | (827) | (556) | (840) | (1,279) | (724) | (557) | (150) | (46) |
| *S. dysgalactiae*,[*] % (n) | 2.37 ± 0.42 | 1.76 ± 0.30 | 1.60 ± 0.26 | 0.22 ± 0.05 | 0.60 ± 0.11 | 0.43 ± 0.09 | 2.10 ± 0.35 | 1.45 ± 0.25 | 0.95 ± 0.31 |
| | (238) | (236) | (232) | (105) | (379) | (139) | (334) | (154) | (45) |
| *Pseudomonas* spp,[*] % (n) | 1.80 ± 0.52 | 3.46 ± 0.93 | 2.85 ± 0.76 | 6.30 ± 1.60 | 3.00 ± 0.80 | 3.25 ± 0.86 | 1.78 ± 0.50 | 2.33 ± 0.63 | 1.71 ± 0.55 |
| | (300) | (817) | (706) | (1,720) | (1,703) | (715) | (531) | (322) | (75) |

[*] Effect of the association with year ($P < 0.001$).

[1] Milk samples submitted to a commercial diagnostic laboratory and cultured according to NMC (2005) guidelines.

[2] Contaminated = growth of 1 or more colonies from 4 or more different microorganisms in culture media. Growth = 3 or more colonies for up to 3 species, except for *Staphylococcus aureus* and *Streptococcus agalactiae* which 1 colony was defined as a growth result. Contagious pathogens = *Staphylococcus aureus, Streptococcus agalactiae* and *Corynebacterium bovis*. Environmental pathogens = coliforms, environmental streptococci, environmental

staphylococci, enterococci, lactococci, and *Pseudomonas* spp. n = represents the number of samples analyzed or the number of positive samples for the respective group or specified pathogen.

[3] Microbiological results of the  most prevalent microorganisms isolated from culture media without the contaminated results representing 65.5% (n = 126,927) milk samples submitted to a commercial diagnostic laboratory from 2012 to 2020. NAS = non-aureus staphylococci.
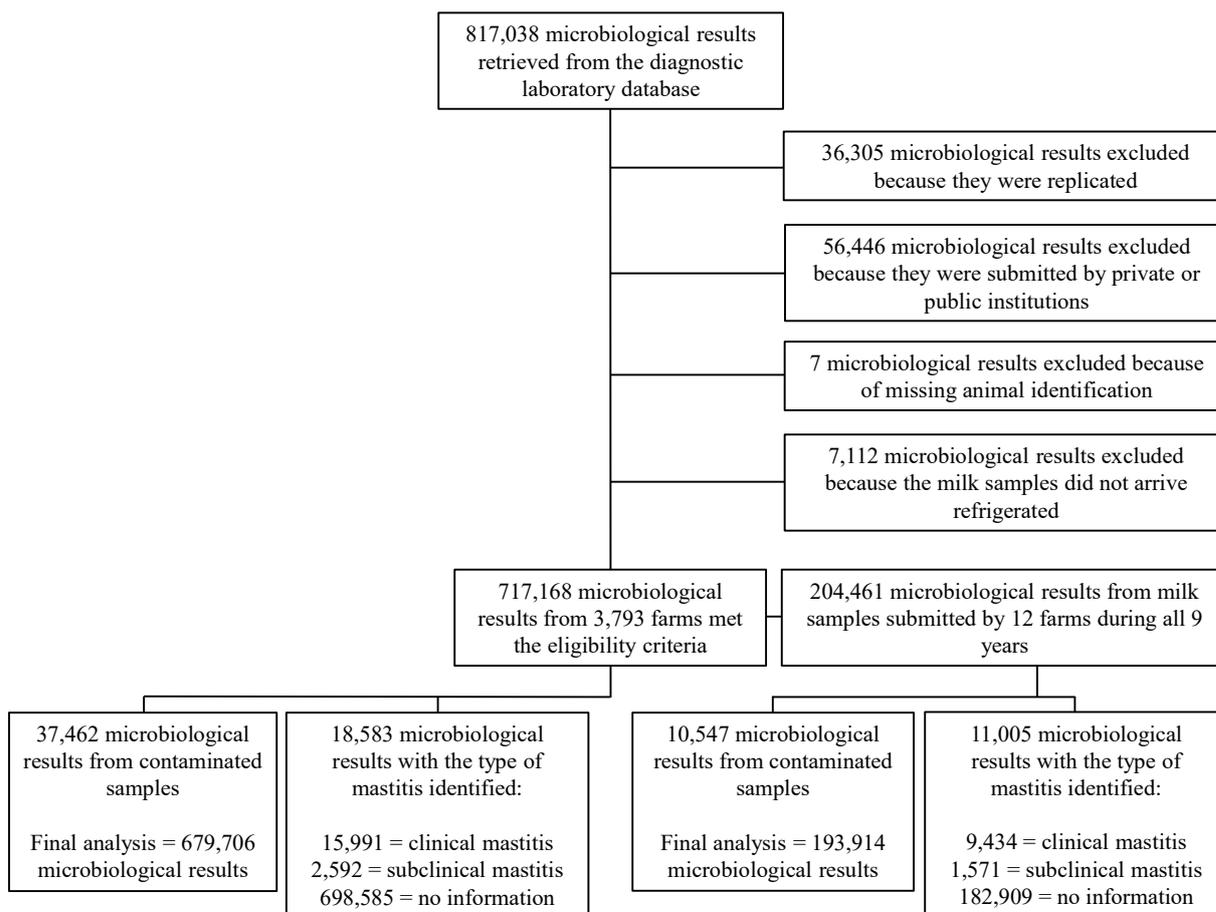
**Figure 1**. Diagram of flow of data retrieved and used for analyses. Milk samples from cows experiencing mastitis or udder health monitoring in Brazilian dairy farms were submitted to a diagnostic laboratory from 2012 to 2020.

**Figure 2**. Proportion of the 18,583 milk samples identified according to the type of mastitis from the 3,793 farms according to the year of submission (A). Pro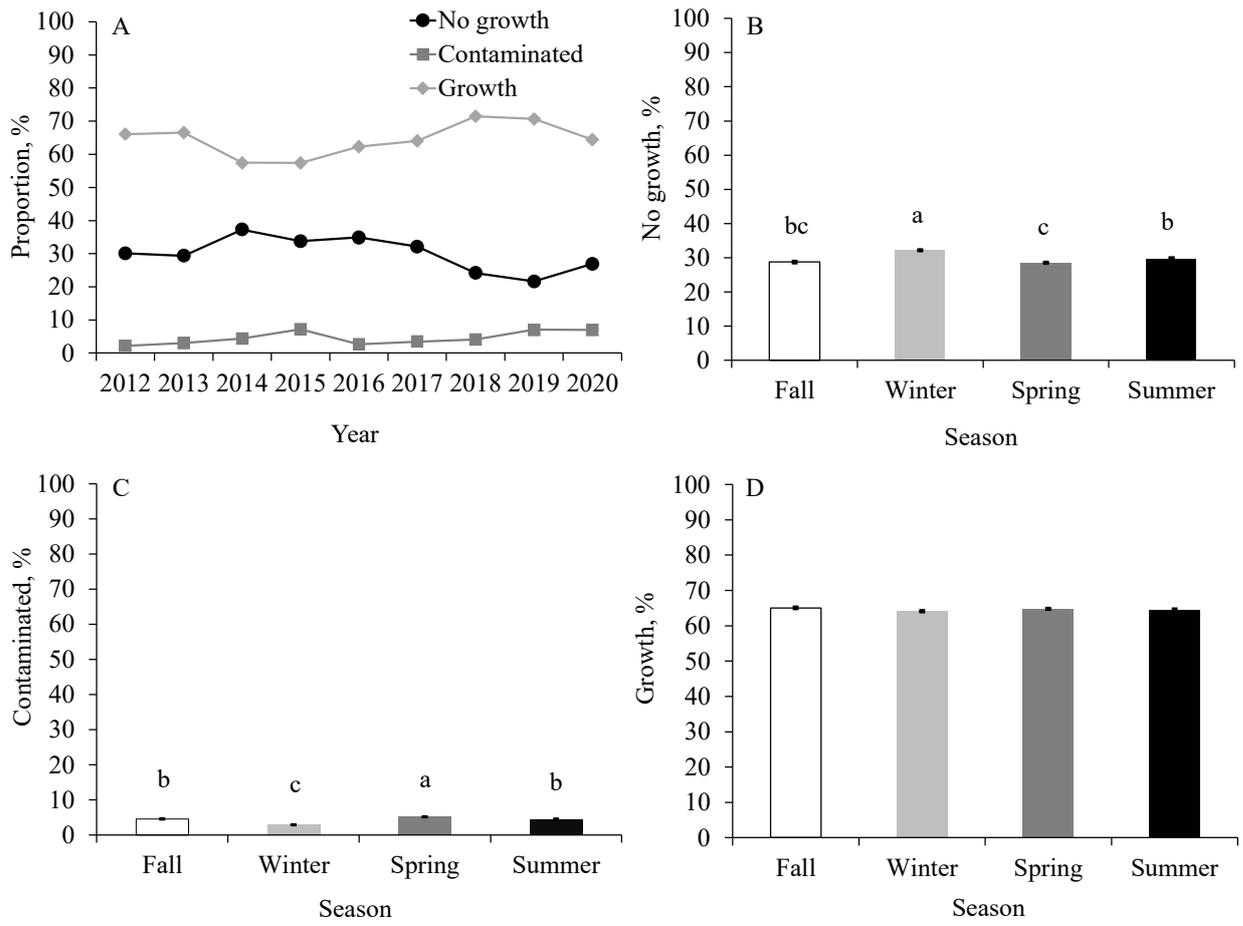portion of the 11,005 milk samples identified according to the type of mastitis from the subset of 12 farms according to year of submission (B).

**Figure 3.** Prevalence of no growth, contaminated, and growth results in milk samples from the 3,793 farms according to the year of submission (A), and the prevalence of no growth (B), contaminated (C), and growth (D) according to season of submission. In panel A, effect of year ($P < 0.001$). In panel B, effect of season ($P < 0.001$) for no growth. In panel C, effect of season ($P < 0.001$) for contaminated. In panel D effect of season ($P = 0.10$) for growth. Error bars represent the standard error mean (SEM). [a,b,c] Within a panel, distinct superscripts denote difference ($P \leq 0.05$).
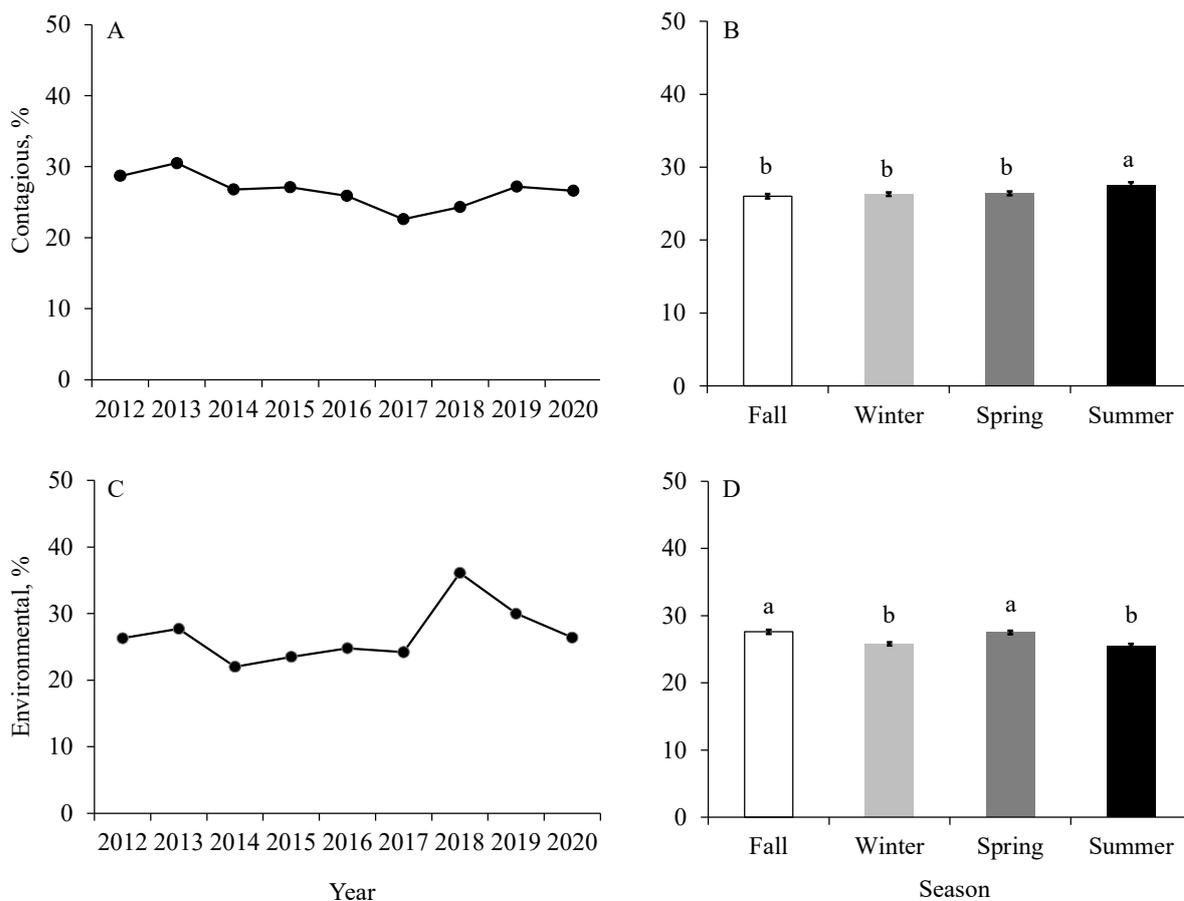
**Figure 4.** Prevalence of contagious pathogens (*Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium bovis*) according to year (A) and season of submission (B), and prevalence of environmental pathogens (coliforms, environmental streptococci, environmental staphylococci, enterococci, lactococci and *Pseudomonas* spp) according to year (C) or season of submission (D) in milk samples from the 3,793 farms. Panel A, effect of year ($P < 0.001$). Panel B, effect of season ($P = 0.002$). Panel C, effect of year ($P < 0.001$). Panel D, effect of season ($P < 0.001$). Error bars represent the standard error of the mean. [a,b,c] Within a panel, distinct superscripts denote difference after adjustment by the method of Tukey ($P \leq 0.05$).
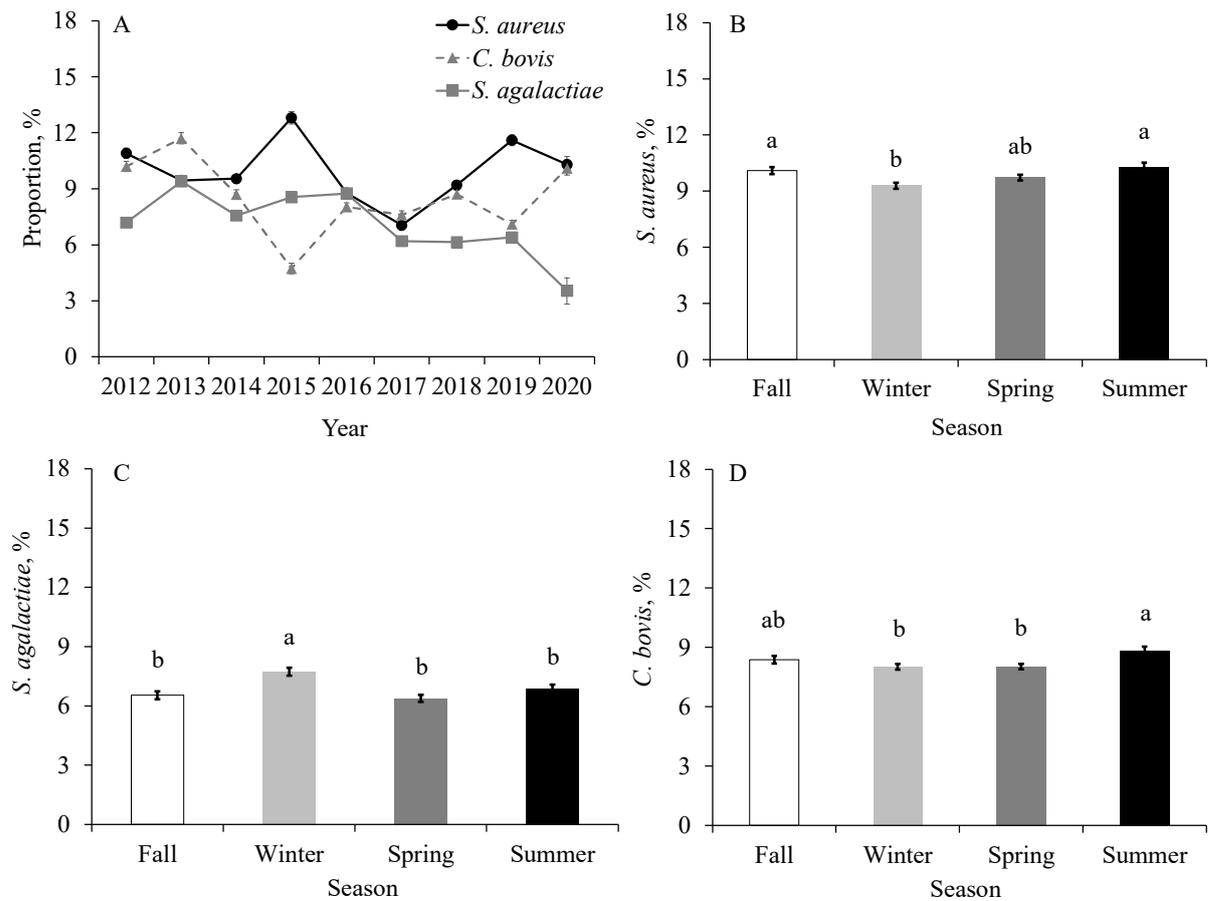
**Figure 5.** Prevalence of the contagious pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae,* and *Corynebacterium bovis*) in milk samples from the 3,793 farms according to year of submission (A), or prevalence of *Staphylococcus aureus* (B), *Streptococcus agalactiae* (C), or *Corynebacterium bovis* (D) according to season of submission. In panel A, effect of year ($P < 0.001$) for the contagious pathogens. In panel B, effect of season ($P < 0.001$) for *Staphylococcus aureus*. In panel C, effect of season ($P < 0.001$) for *Streptococcus agalactiae*. In panel D, effect of season ($P < 0.001$) for *Corynebacterium bovis*. Error bars represent the standard error mean (SEM). [a,b,c] Within a panel, distinct superscripts denote difference ($P \leq 0.05$).
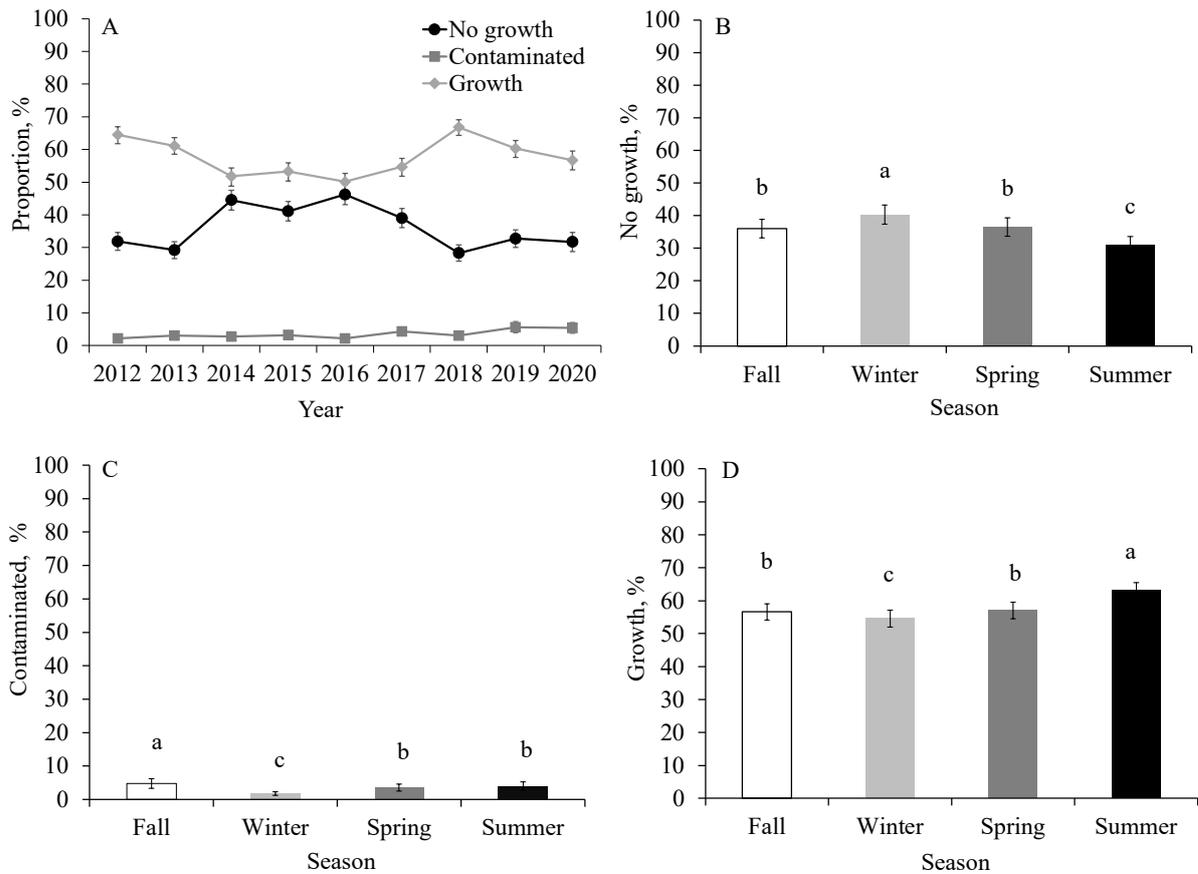
**Figure 6.** Prevalence of no growth, contaminated, and growth results in milk samples from the 12 farms according to the year of submission (A), and the prevalence of no growth (B), contaminated (C), and growth (D) according to season of submission. Panel A, effect of year ($P < 0.001$). Panel B, effect of season ($P < 0.001$). Panel C, effect of season ($P < 0.001$). Panel D effect of season ($P < 0.001$). Error bars represent the standard error of the mean. [a,b,c] Within a panel, distinct superscripts denote difference after adjustment by the method of Tukey ($P \leq 0.05$).
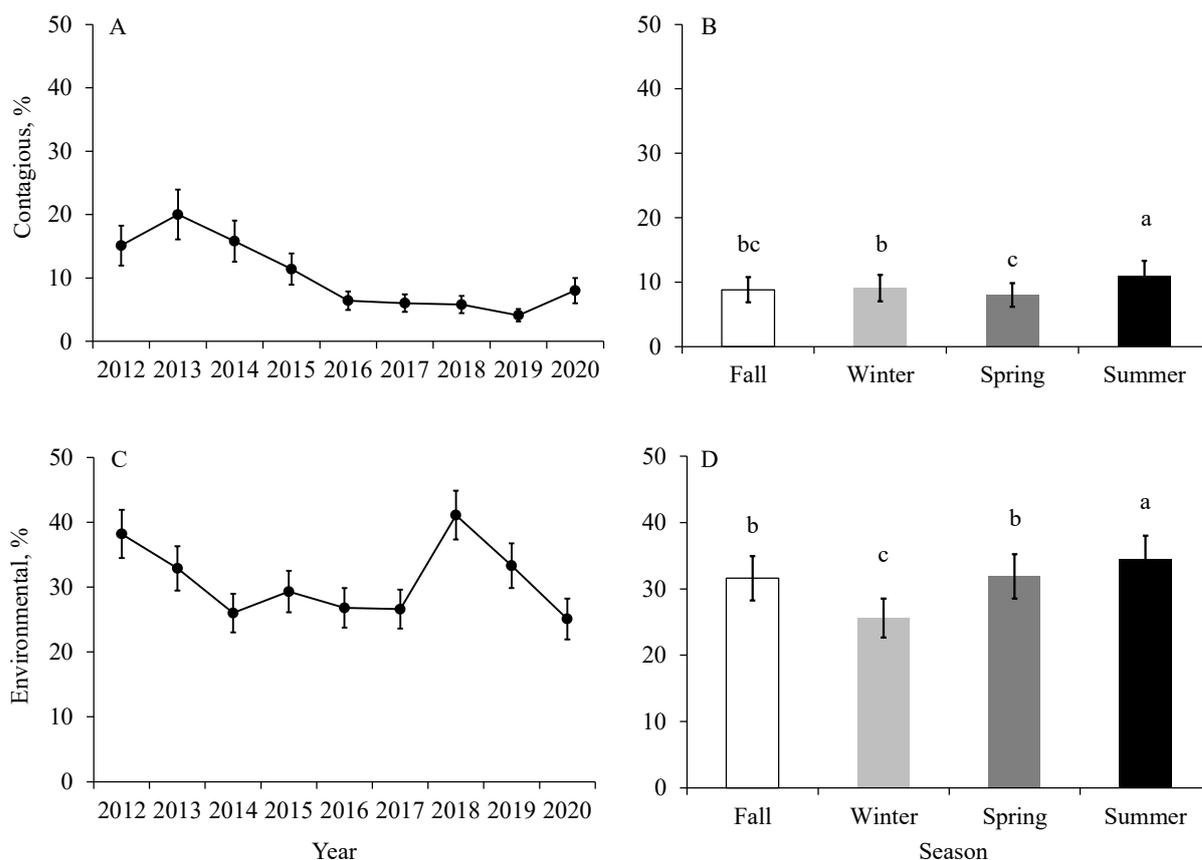
**Figure 7.** Prevalence of contagious pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae,* and *Corynebacterium bovis*) in milk samples from the 12 farms according to year of submission (A), or prevalence of *Staphylococcus aureus* (B), *Streptococcus agalactiae* (C), or *Corynebacterium bovis* (D) according to the season of submission. In panel A, effect of year ($P < 0.001$) for the contagious pathogens. In panel B, effect of season ($P = 0.22$) for *Staphylococcus aureus*. In panel C, effect of season ($P < 0.001$) for *Streptococcus agalactiae*. . In panel D, effect of season ($P < 0.001$) for *Corynebacterium bovis*. Error bars represent the standard error mean (SEM). [a,b,c] Within a panel, distinct superscripts denote difference ($P \leq 0.05$).
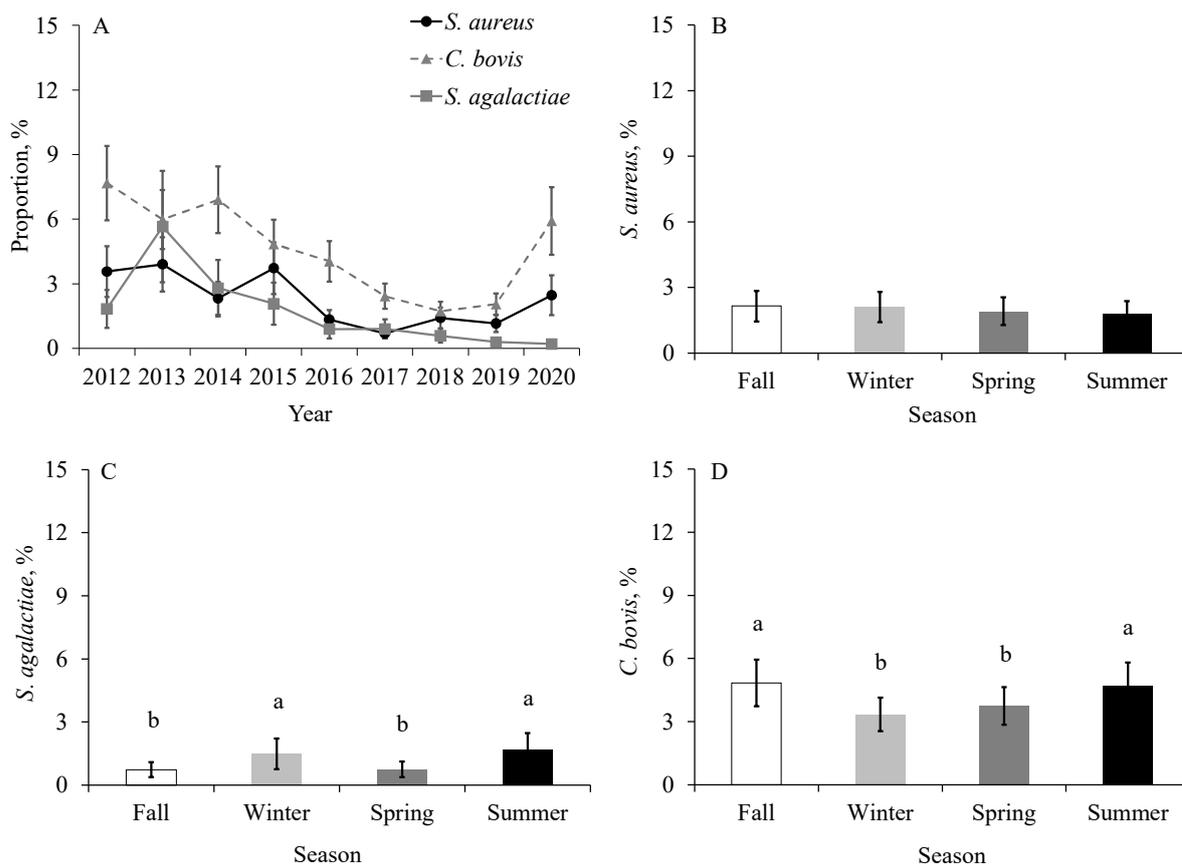
**Figure 8.** Prevalence of specified contagious pathogens *Staphylococcus aureus*, *Corynebacterium bovis*, and *Streptococcus agalactiae* in milk samples from the 12 farms according to year (A) of submission, or prevalence of *Staphylococcus aureus* (B), *Streptococcus agalactiae* (C) or of *Corynebacterium bovis* (D) according to the season of submission. Panel A, effect of year for *S. aureus* ($P < 0.001$), *C. bovis* ($P < 0.001$) and *S. agalactiae* ($P < 0.001$). Panel B, effect of season ($P = 0.22$). Panel C, effect of season ($P < 0.001$). Panel D, effect of season ($P < 0.001$). Error bars represent the standard error of the mean. [a,b,c] Within a panel, distinct superscripts denote difference after adjustment by the method of Tukey ($P \leq 0.05$).

# IMPLICAÇÕES

Está bem estabelecido que a mastite seguirá sendo uma das doenças mais relevantes para as vacas leiteiras, uma das mais difíceis de ser controlada e sua erradicação é praticamente impossível por ser causada por inúmeros patógenos com características microbiológicas diversas, presentes na glândula mamária e no ambiente em que a vaca vive, perfil muitas vezes altamente contagioso, e alguns apresentando cronicidade. Não só isso, ao oposto de outras doenças que têm um período de risco mais definido, mastite é uma doença que pode ocorrer toda vez que a vaca é ordenhada, ou seja, diariamente durante toda lactação.

Conhecer o perfil dos patógenos mais prevalentes no rebanho leiteiro é essencial para definir as medidas de controle, prevenção e tratamento específicas para cada patógeno causador de mastite.

Apesar do estudo apresentado nesta dissertação utilizar uma amostragem conveniente de rebanhos no Brasil e da maioria destes rebanhos não ter submetido amostras de leite em todos os 9 anos, ele inclui 3.793 fazendas e mais de 717 mil resultados microbiológicos de cultura de leite, representando as 5 regiões geográficas do país num período de 9 anos. Tal amostragem nos permite inferir sobre a situação atual dos agentes bacterianos de mastite no rebanho nacional e as eventuais mudanças ao longo do período estudado de 2012 a 2020.

Os resultados nos permitem concluir que, no Brasil, uma pequena redução na prevalência de patógenos de mastite considerados contagiosos foi observada, porém esta redução não foi suficiente para observamos uma mudança no perfil de patógenos contagiosos para o perfil ambiental o que demonstra que as fazendas produtoras de leite no Brasil ainda não evoluíram do ponto de vista de controle de mastite da mesma forma que já foi observada em outros países. Ou seja, há necessidade de se implementar medidas de controle, tratamento e prevenção específicas para erradicar agentes como o *S. agalactiae* e *C. bovis* como primeiro passo, e controlar o *S. aureus* nos rebanhos leiteiros brasileiros de forma conjunta e síncrona de todas as pessoas envolvidas na produção de leite no país. Um subconjunto de 12 fazendas comprometidas com a identificação de patógenos causadores de mastite reduziu a prevalência de *S. aureus, S. agalactiae* e *C. bovis* de suas vacas leiteiras, e uma mudança de perfil de patógenos causadores de mastite contagiosa para ambiental foi observada. Isto comprova que os esforços focados na

erradicação e controle destes patógenos pelos produtores que estão comprometidos a longo prazo com a identificação de patógenos específicos e provavelmente implementam medidas de controle específicas são bem-sucedidos, reduzem os prejuízos econômicos da mastite, produzem um leite de melhor qualidade e têm maior rentabilidade na atividade.

Comparação com outros estudos epidemiológicos publicados mundialmente pode ser difícil por diversas razões como, diferenças nas técnicas de coleta e critérios usados nos diagnósticos microbiológicos, nas condições climáticas e de sistemas de produção de cada país, agrupamento das bactérias, análises estatísticas e duração do período estudado. No entanto, eles demonstram que os rebanhos, regiões e países têm constantes desafios e diferentes perfis e prevalências de patógenos que mudam ao longo dos anos, principalmente, como consequência das ações implementadas em nível regional ou nacional.

É importante ressaltar que este estudo permitiu associar as prevalências dos principais patógenos aos anos e estação de ano, porém por ser descritivo e não mecanístico, explicações sobre causa e efeito não podem ser determinadas. Apesar de associativo, as inferências deste estudo podem contribuir com o trabalho dos produtores de leite, dos consultores de qualidade do leite, das cooperativas, das autoridades sanitárias, com futuras pesquisas científicas, e com desenvolvimento de novas soluções mercadológicas para controle e tratamento da mastite no Brasil.