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LISIANE DA SILVEIRA GARCIA

FATORES QUE AFETAM A ESTABILIDADE DO LEITE CRU BOVINO: UMA REVISÃO SISTEMÁTICA

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Dissertação apresentada como requisito para obtenção do Grau de Mestre em Zootecnia, na Faculdade de Agronomia, da Universidade Federal do Rio Grande do Sul.

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minha família, que muito me apoiou
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FATORES QUE AFETAM A ESTABILIDADE DO LEITE CRU BOVINO[1]: UMA REVISÃO SISTEMÁTICA

Autor: Lisiane da Silveira Garcia

Orientador: Vivian Fischer

Co-orientador: Ines Andretta

RESUMO - A presente dissertação traz, no capítulo I, uma revisão bibliográfica feita sobre os principais fatores que afetam a estabilidade do leite cru bovino, com a apresentação do mapa conceitual elaborado pela autora durante a idealização do projeto da presente dissertação, junto com os objetivos e hipóteses. No capítulo II foram relaciodos os resultados da revisão sistemática da literatura, proposta após a realização da revisão bibliográfica simples, ao se verificar que não havia um trabalho relacionando os vários fatores mencionados como agentes que afetam a estabilidade do leite cru bovino. Nesta seção são apresentados os resultados das frequências dos fatores mais mencionados na literatura e as graduações alcoólicas mais utilizadas nos testes do álcool ao redor do mundo. Além disso, se destacaram os países que mais publicaram sobre o tema, mostrando as regiões do globo em que o problema da baixa estabilidade do leite é mais recorrente. Após a análise dos resultados foi elaborado um segundo mapa conceitual mais completo, reflexo da revisão de todos os achados da revisão sistemática. Os capítulos III e IV trazem o resultado das análises estatísticas quantitativas dos dois principais fatores que afetam a estabilidade do leite mencionados na revisão sistemática: estação do ano em que os dados extraídos dos artigos foram reanalisados; e a restrição alimentar apresentada em forma de meta-análise (análise conjunta bruta dos dados), respectivamente. Ao final foram apresentadas as conclusões finais após apreciação e síntese de todos os resultados dos capítulos anteriores.

[1] Dissertação de Mestrado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (145p.) Março, 2022.

FACTORS AFFECTING THE STABILITY OF RAW BOVINE MILK[2]

Author: Lisiane da Silveira Garcia

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ABSTRACT - This dissertation presents, in Chapter I, the bibliographic review made on the main factors that affect the stability of raw bovine milk, showing the conceptual map elaborated by the author during the idealization of the project of this dissertation, together with the objectives and hypotheses sought after the completion of the stages of this work. Chapter II relates to the results of the systematic review of the literature, proposed after the simple literature review, when it was found that there was no work relating the various factors mentioned as agents affecting the stability of raw bovine milk. This section presents the statistical results of the frequencies of the most mentioned factors in the literature and of the most used alcoholic grades in alcohol tests around the world. In addition, it highlighted the countries that published the most on the subject, showing the regions of the world where the problem of low milk stability is more recurrent. After the analysis of the results, a second, more complete conceptual map was elaborated, reflecting the review of all the findings of the systematic review. Chapters III and IV contain the results of the quantitative statistical analyses of the two main factors affecting milk stability according to the systematic review, the season of the year, which underwent a review of the data extracted from the articles; and the feed restriction presented as a meta-analysis (gross joint data analysis), respectively. Finally, the final conclusions are presented after consideration and synthesis of all the results of the previous chapters.

[2] Master of Science Dissertation in Animal Production, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (145p.) March, 2022.

SUMÁRIO

LISTA DE FIGURAS

CAPÍTULO I

CAPÍTULO II

CAPÍTULO III

CAPÍTULO IV

LISTA DE TABELAS

LISTA DE ABREVIATURAS

αs1- Alfa 1 caseína

αs2- Alfa 2 caseína

β - Beta caseína

κ - Kapa caseína

ɣ - Gama caseína

β-LG - Beta-lactoglobulina

BEN - Balanço Energético Negativo

CBT - Contagem Bacteriana Total

CCS - Contagem de Células Somáticas

CPP - Contagem Padrão em Placa

DAD - Diet Anionic

°GL - Graduação alcoólica

HIUS - High Intensity Thermal Ultrasound

HP - High Pressure

LINA - Leite Instável Não Ácido

LP - lactoperoxidase

MES - Milk Ethanol Stability

NDP - Non-rumen Degradable Protein

RDP - Rumen Degradable Protein

SCC - Somatic Cell Count

TDN - Total Digestible Nutrients

Tgase - Transglutaminase

UAT - Ultra Alta Temperatura

UHT - Ultra High Temperature

UNAM - Unstable Non-acid Milk

CAPÍTULO I

1.INTRODUÇÃO

O interesse em estudar os fenômenos que afetam a estabilidade do leite se justifica por inúmeras transformações dessa matéria-prima ao ser processada em outros produtos derivados como leite Ultra Alta Temperatura (UAT), leite condensado, queijos, etc. O componente lácteo mais relacionado com a estabilidade é sua porção protéica, mais precisamente, as micelas de caseína. As caseínas são as principais proteínas do leite e consistem de quatro proteínas principais: αs1-, αs2-, β- e κ-caseína e representam aproximadamente 80% da proteína total no leite de bovinos e de outras espécies leiteiras comerciais (Fox e Brodkorb, 2008).

O leite deve ser processado termicamente antes de seu consumo. As caseínas, principais proteínas lácteas, apresentam propriedades muito interessantes em se manterem em dispersão coloidal mesmo durante e após o processamento térmico, mantendo a fluidez e integridade dos componentes assim como em condições mais ácidas e sob temperatura moderada, podendo coagular de forma controlada e desejável, processo essencial na produção de queijos (Fox et al., 2015). Segundo Singh & Creamer (1992), as frações proteicas β-lactoglobulina (β-LG) e κ-caseína (κ-CN), possuem uma grande influência na estabilidade do leite ao calor.

Essa habilidade do leite em manter a sua estrutura é denominada de estabilidade. Muitos fatores afetam a estabilidade do leite e sua integridade, desde sua síntese pelo animal até sua chegada na indústria. A estabilidade da proteína do leite pode ser definida como a capacidade da caseína em se manter em suspensão coloidal e da lactoalbumina e lactoglobulina em permanecerem em solução quando o leite é submetido ao calor ou outras influências modificadoras como o aumento do cálcio iônico e a diminuição do pH (White & Davies 1958). Existem várias maneiras de estimar a estabilidade do leite. Uma delas é a estabilidade ao etanol, a qual foi definida como a mínima concentração de etanol aquoso adicionado que dá origem à coagulação do leite (Horne & Parker, 1980). A estabilidade do leite cru é um assunto relevante para fins de uso industrial do leite e para atender aspectos de legislação do Brasil e de outros países (Horne, 2016).

O leite, após a ordenha, é refrigerado em tanque de expansão, e armazenado por até 48 horas, como consta nas Instruções Normativas n° 76 e 77 do Ministério de Agricultura, Pecuária e Abastecimento (Brasil, 2018[a,b]). Em alguns casos há a necessidade de o leite ser coletado da propriedade e ser armazenado em um entreposto antes de se dirigir para a indústria que o processa. As modificações do leite desde a sua secreção, ordenha, armazenagem na fazenda e transporte até a indústria podem afetar a sua composição, alterando a estabilidade do leite.

Existem diversos estudos que avaliaram os efeitos de práticas de alimentação ou níveis nutricionais (Gabbi et al., 2016, 2018), estresse térmico (Abreu et al., 2011), estádio de lactação (Horne, 1986), duração da lactação (Marques et al., 2010), doenças digestivas e metabólicas (Marques et al., 2011; Fagnani et 2014; Werncke, 2017), temperatura de armazenamento do leite em tanques refrigeradores nas propriedades rurais (Reche et al., 2016) e agitação mecânica do leite sobre a estabilidade do leite (Warmiñska et al., 2003). De modo geral, esses estudos evidenciaram que o aporte nutricional insuficiente e/ou desequilibrado, estresse térmico, estádio de lactação muito inicial ou muito tardio, ocorrência de doenças digestivas e metabólicas reduzem a estabilidade do leite. Além disso, tempo excessivo de armazenamento, CPP elevada e temperatura marginal de resfriamento podem provocar a elevação da acidez que mesmo dentro da faixa aceitável (14-18°D), reduzem a estabilidade do leite (Reche, 2016).

O leite pode percorrer longas distâncias ao sair da propriedade até chegar à indústria. Muitas vezes, o caminho percorrido apresenta estradas em más condições e essas condições desfavoráveis nas vias submetem o leite a fortes agitações dentro do compartimento de transporte. O leite submetido a diferentes faixas de agitação tem suas características qualitativas alteradas, como, por exemplo, a diminuição da estabilidade térmica (Warmiñska et al., 2003). Além disso, a agitação pode aumentar a lipólise do leite (Deeth & Fitzgerald,1976).

O objetivo deste estudo foi elaborar uma revisão sistemática sobre os fatores que de fato afetam a estabilidade do leite comprometendo sua resistência aos tratamentos térmicos e transformações na indústria, diminuindo as perdas do produtor e também da indústria com a manutenção da sustentabilidade da produção leiteira, visto que esse problema causa impactos pelo descarte do produto instável na maioria das vezes.

2.REVISÃO BIBLIOGRÁFICA

2.1 Revisão Sistemática e Meta-análise

As revisões sistemáticas e meta-análise têm sido amplamente utilizadas na área científica e acadêmica a fim de diminuir a replicação de trabalhos semelhantes e elaborar um panorama geral e objetivo sobre o que se sabe e o que falta saber sobre determinado assunto. A revisão sistemática da literatura disponível é uma ferramenta utilizada para minimizar os erros. Consiste em uma busca em mais de dois bancos de dados confiáveis em que se monta uma chave de busca em cima do acrônimo PICO, que significa P - população; I - interesse; C - intervenção e O - resultado, com o objetivo da pesquisa a ser realizada. A revisão sistemática pode ou não incluir meta-análise (Egger et al., 2001). A meta-análise pode ser utilizada para analisar dados de diversos estudos que abordam a mesma questão de pesquisa. Os dados então são combinados, gerando assim uma estimativa que resume o todo (Rodrigues & Ziegelmann, 2010). A primeira meta-análise da história é atribuída ao estatístico Karl Pearson, que em 1904 combinou, através de correlações, os dados de cinco estudos para examinar o efeito preventivo de inoculações contra febre entérica (Pearson, 1904).

2.2. O leite

Segundo a legislação brasileira Decreto n° 9.013, se entende por leite, sem outra especificação, o produto oriundo da ordenha completa, ininterrupta, em condições de higiene, de vacas sadias, bem alimentadas e descansadas (BRASIL, 2017). Segundo Fernández et al. (2015), o leite de vaca é um alimento básico da dieta humana e faz parte de nossa dieta há pelo menos 10.000 anos. É considerado um alimento completo e balanceado, proporcionando alto teor de nutrientes em relação ao teor calórico: fornece proteínas de alto valor biológico, carboidratos (principalmente na forma de lactose), gorduras, vitaminas do complexo B e minerais, especialmente cálcio e fósforo(Foods Standards Agency, 2002). De acordo com Raikos & Dassios (2014), graças à alta digestibilidade das proteínas do leite, nos últimos anos, foram descritos fragmentos de aminoácidos advindos das proteínas do leite de vaca em proteínas do corpo humano. Eles parecem ter uma atividade específica no nível gastrointestinal e sistêmico como imunomoduladores e propriedades antimicrobiana, antihipertensiva e antitrombótica. O leite e outros produtos lácteos são uma fonte importante de macro e micronutrientes em todas as fases da vida humana, mas principalmente na infância e adolescência, e desempenham um papel importante no cumprimento das recomendações de ingestão atuais destes nutrientes (Nicles et al., 2009).

2.3. Leite Instável não ácido (LINA)

A estabilidade térmica do leite pode ser definida como o tempo necessário para ocorrer coagulação visível, em determinado pH e temperatura e está diretamente relacionada à capacidade do leite em resistir à coagulação em condições de elevada temperatura, usualmente 140 ºC (Horne & Muir, 1990).

Há duas situações que o leite pode se tornar instável:

(1) alta contagem bacteriana, levando a formação de ácidos orgânicos.

(2) etiologia desconhecida, designada como Leite Instável Não Ácido (LINA), cuja acidez é normal, mas ele coagula ao teste do álcool mesmo sem estar ácido.

Com relação à estabilidade do leite, a legislação atual (Instrução Normativa n° 76) estabelece como padrão a estabilidade ao etanol 72% (v/v), a qual representa uma medida rápida e eficiente de avaliação da estabilidade térmica. Segundo Zanela et al. (2009), a grande maioria das indústrias utiliza o teste de estabilidade ao etanol em concentrações superiores, o que pode levar ao descarte de leite de forma injustificada.

2.4. Estabilidade do leite e as caseínas

A estabilidade do leite está ligada à conformidade das caseínas do leite. As caseínas são as principais proteínas do leite, compreendendo aproximadamente 80% das proteínas lácteas e consistem de quatro proteínas principais: αs1-, αs2-, β- e κ-caseína (Figura 1), as quais possuem elevada estabilidade térmica, o que permite às indústrias de laticínio realizar o tratamento do leite a temperaturas elevadas, como o leite submetido à Ultra Alta Temperatura (UAT).

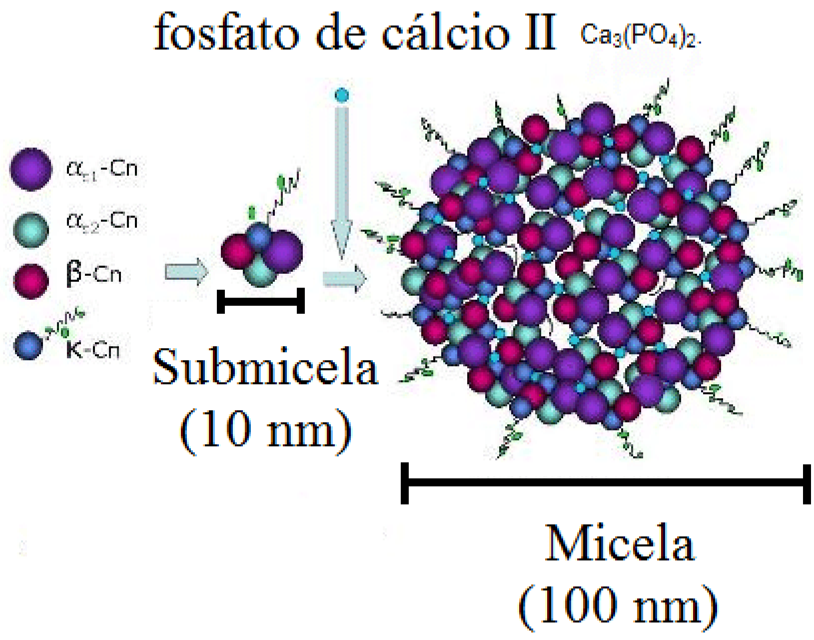


Figura 1. Submicela e micela de caseína
Fonte: Test Kappa, 2010

Segundo Fortuna (2015), as caseínas diferem entre si pela sua composição de aminoácidos, que lhes conferem características particulares:

a) caseína αs-1 – constituída por cadeias polipeptídicas, três regiões da sua sequência primária são formadas por resíduos com cadeias laterais apolares, ou hidrofóbicas, situadas entre os resíduos de aminoácidos 1 - 44, 90 – 133, 132 – 199. Apresenta uma região polar ácida, que contém muitos resíduos de aminoácidos carregados, localizados entre os resíduos 41 - 80, onde se localizam sete dos oito resíduos fosforilserina e, onde se concentra a carga líquida da proteína (Sgarbieri, 2005). Esta proteína é considerada fraca e flexível (Oliveira & Timm, 2007), permitindo a entrada de proteases e precipitando com níveis de cálcio muito baixos. Outro fato é a ausência do aminoácido cisteína em sua estrutura molecular (Cheftel et al. 1989).

b) caseína αs-2 – possui uma estrutura bipolar, os resíduos fosforil em número de 10 a 13 estão agrupados em três segmentos, 8 - 16, 56 - 61, 129 - 133. Já os resíduos apolares se encontram nas porções 90 - 120 e 160 - 207 (sequência carboxiterminal). Apresenta dois resíduos de cisteína por molécula, sendo a mais fosforilada e a mais rica em resíduos catiônicos (Sgarbieri, 2005). Possui uma grande sensibilidade aos íons , Ca^{2+} por isso está mais suscetível a precipitação por este íon do que a caseína αs1 (Walstra & Jenness, 1984).

c) caseína β – na presença de íons Ca^{2+} formam suspensões coloidais ao invés de precipitarem como as caseínas αs-1. É uma proteína anfipática com uma região hidrofílica na porção N-terminal e uma parte carboxiterminal localizada nos resíduos 136 – 209. Estes resíduos na grande maioria são apolares, no entanto, as regiões entre os resíduos de 1 a 135 possuem cinco resíduos fosforil, contendo toda a carga líquida da proteína. O conteúdo de prolina é relativamente alto e é a mais hidrófoba das caseínas (Sgarbieri, 2005). A β- caseína é dependente da temperatura, pois em 20 °C forma grandes polímeros, mas a 4 ºC não possui esta propriedade (Oliveira & Timm, 2007), podendo perder a estrutura micelar e se tornar mais sensível à ação das proteases (Cheftel et al., 1989). A estrutura primária da β-caseína é facilmente hidrolisada pela protease plasmina nas ligações peptídicas dos resíduos de aminoácidos 28 - 29, 105- 106 e 107 - 108, produzindo fragmentos peptídicos chamados caseínas γ, que se difundem para o soro, constituindo uma parte da fração proteose-peptona (Sgarbieri, 2005).

d) κ-caseína – é uma glicoproteína, possuindo um grupo fosfoserina, é estável na presença dos íons de cálcio, assumindo um papel importante na estabilidade das micelas. O fosfato de cálcio atua como um agente cimentante, mas se não houver κ- caseína, a agregação continuará até a formação de um gel ou de um precipitado (Oliveira & Timm, 2007). Esta micela apresenta dois resíduos de cisteína α por molécula, só apresenta um resíduo fosforilado, fixa apenas alguns íons Ca^{2+} e sua solubilidade não é afetada pela sua presença (Cheftel et al., 1989). Sgarbieri (2005) relata que a hidrólise enzimática que ocorre no processamento do queijo ou no tratamento térmico em temperaturas elevadas resulta na remoção ou dissociação da κ-caseína eliminando a estabilização eletrostática e estérica da superfície micelar, aumentando a hidrofobicidade de superfície, o que resulta em agregação das micelas e formação de coágulo;

e) caseína γ - resulta da proteólise da caseína β. A estrutura exata das micelas de caseína ainda permanece em debate, alguns modelos têm sido sugeridos por diversos autores (Fox & Mcsweeney, 1998; Walstra, 1999).

No entanto, alguns fatores como a hidrólise enzimática da k-caseína, temperatura, pH, excesso de cálcio e adição de etanol afetam a estabilidade dessas proteínas, que estão em grande parte presentes no leite na forma de partículas coloidais, conhecidas como micelas (Fox e Brodkorb, 2008).

2.5. Formas de avaliação da estabilidade do leite

A estabilidade do leite, ou seja, a resistência do leite ao aquecimento pode ser avaliada de várias maneiras, como teste da fervura, teste do álcool ou etanol ou alizarol e teste da estabilidade térmica (Chavez et al., 2004; Machado et al., 2017). A estabilidade do leite ao etanol foi definida como a mínima concentração de etanol aquoso adicionado que coagula o leite (Horne & Parker, 1979).

O valor mínimo da estabilidade exigido pela legislação e/ou indústrias varia entre países e estabelecimentos. O valor de 68% é aceito em países da África (Kassa et al., 2013; Rathnayake et al, 2016), enquanto o valor de 70% é aceito em países como Chile, Uruguai e Argentina (Chavez et al., 2004; Barros et al., 2001). No Brasil, perante a legislação (Instrução Normativa n° 76, 2018), a graduação alcoólica estabelecida é 72% v/v, mas sabe-se que as indústrias exigem dos produtores valores que oscilam entre 76 e 82 °GL.

O pressuposto do teste é que se um volume de leite pré-estabelecido reagir com uma quantidade de álcool e coagular, esse leite não apresenta estabilidade suficiente para resistir aos tratamentos térmicos na indústria, muiras vezes em função do excesso de acidez adquirida, por causa da fermentação bacteriana. Há também outras maneiras de avaliar a estabilidade do leite cru bovino. O teste da fervura também detecta a formação de grumos em leite ácido, pois está relacionado ao pH do leite. O leite com suas características estáveis a tratamentos térmicos não forma grumos (Brasil, 2018). Entretanto, é um teste muito trabalhoso e despende muito mais tempo para fazer as análises na indústria e até mesmo na unidade produtiva, o que o inviabiliza.

Hatting (2017) analisou técnicas de detecção de coágulo no leite. Foram avaliados o teste do Alizarol, o ensaio de protease, a técnica da placa de ágar, RPHPLC e o software MILQC. O teste Alizarol detecta a floculação do leite de forma eficaz, no entanto, os resultados são baseados na interpretação pelo olho humano. São necessários mais trabalhos que possam testar outros testes baratos e rápidos na detecção da estabilidade do leite à floculação.

2.6. Variação da estabilidade do leite

A estabilidade do leite varia entre os meses do ano, em parte relacionadas com variação no aporte de alimentos e nas condições climáticas. Valores baixos e altos de estabilidade do etanol observados em meses diferentes dependendo do ano podem ser parcialmente explicados pela variação no fornecimento ou qualidade da ração com consequentes mudanças na composição mineral (Tsioulpas et al., 2007; Lewis 2011; Stumpf et al., 2013; Horne, 2015).

Há registros de maior incidência de leite instável não ácido nos meses de verão, onde há menor aporte de matéria-seca e maior susceptibilidade ao estresse térmico (Barchiesi-Ferrari et al., 2007; Gaucher et al., 2008). Alguns estudos relataram efeitos significativos na estabilidade relacionados à composição da caseína (Barbosa et al., 2012), enquanto outros não descreveram efeitos significativos (Marques et al., 2011; Botaro et al., 2007, 2009). A Contagem de Células Somáticas (CCS) tem sido controversamente relacionada à baixa estabilidade, pois alguns estudos relataram relação entre alta CCS e baixa estabilidade (Oliveira et al., 2013), enquanto outros não mostraram nenhuma relação (Kolling, 2012).

2.7. Fatores que afetam a estabilidade do leite cru

A estabilidade do leite, que é a estabilidade das moléculas de caseína, pode ser influenciada por diversos fatores, ligados ou não ao animal, como mostra o mapa conceitual abaixo.

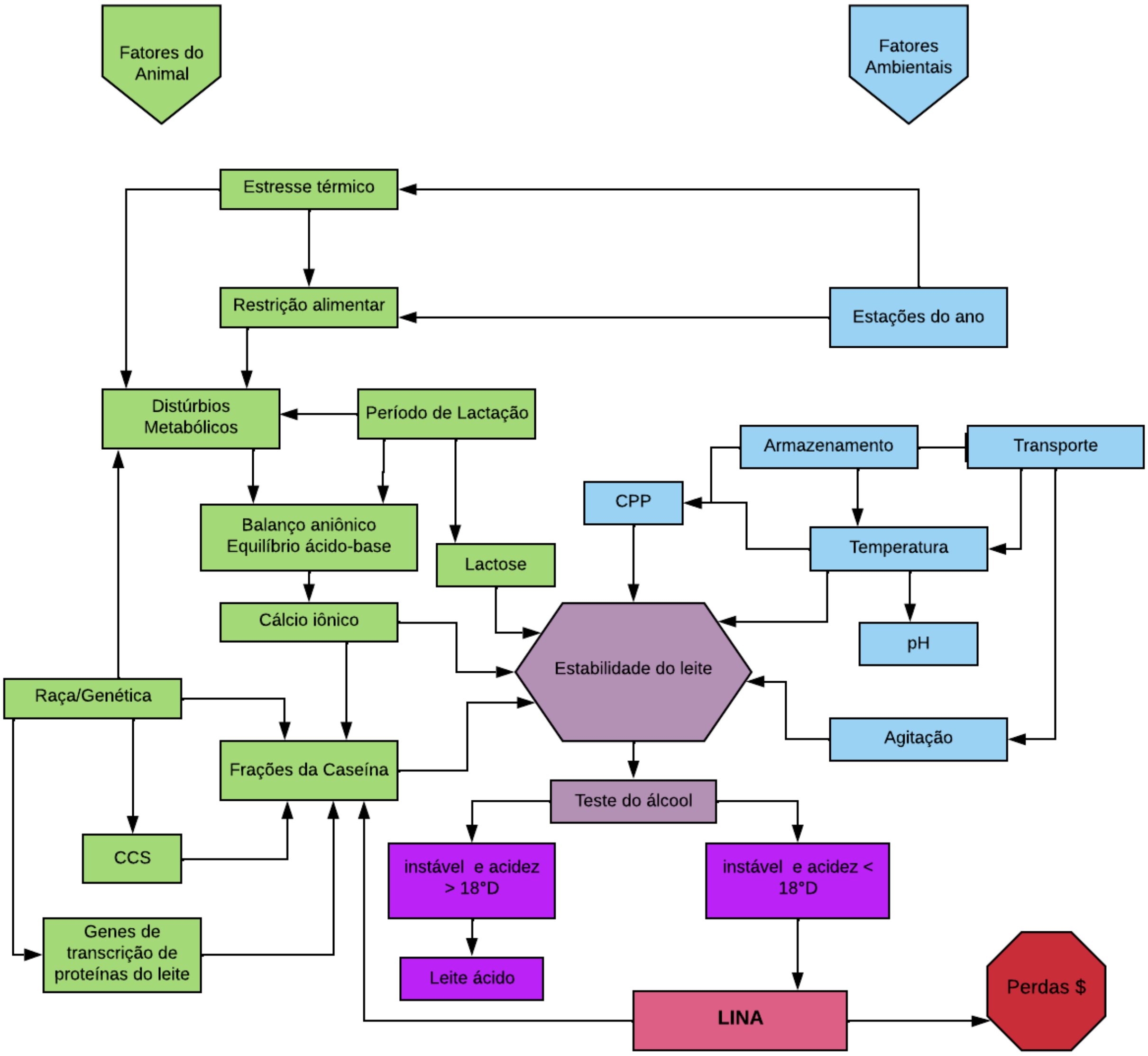


Figura 2. Mapa mental sobre os fatores que afetam a estabilidade do leite preliminar
Fonte: Autora

2.7.1. Fatores ligados à composição do leite

A estabilidade do leite é uma questão complexa (Horne, 2015), e algumas características do leite têm sido consistentemente relacionadas à baixa estabilidade do etanol, como acidez adquirida elevada (Rathnayake et al., 2016), concentração de cálcio iônico (Lewis, 2011; Tsioulpas et al., 2007), força iônica (Chavez et al., 2004), fosfato (Gaucheron, 2005) e conteúdo de citrato (Tsioulpas et al., 2007). No caso do leite, a sua estabilidade está ligada à estrutura micelar da caseína, sendo essa estruturação que mantém uma força de repulsão que impede a coagulação das caseínas. A estabilidade do leite está negativamente relacionada à acidez, pois a redução do pH reduz o caseinato de fosfato de cálcio e aumenta a concentração de cálcio iônico, que por sua vez, diminui as forças de repulsão entre as caseínas, favorecendo a coagulação (Lewis, 2011; Horne, 2015). Horne e Muir (1990), Chavez et al. (2004) e Lewis (2011) relataram que a estabilidade do leite foi adversamente afetada pela concentração de cálcio iônico. A saída de fosfato de cálcio da estrutura micelar leva à exposição da porção hidrofóbica das caseínas, aumentando a propensão para a agregação (Philippe et al. 2005).

Alguns autores relataram efeitos significativos na estabilidade relacionados à composição da caseína (Barbosa et al., 2012), enquanto outros não os observaram (Marques et al., 2011; Botaro et al., 2007, 2009). A contagem de células somáticas (CCS) tem sido controversamente relacionada à baixa estabilidade, pois alguns estudos relataram relação entre alta CCS e baixa estabilidade (Oliveira et al., 2013), enquanto outros não mostraram nenhuma relação (Kolling, 2012).

No trabalho de Walstra (1999), o autor constatou que a caseína é formada por submicelas constituídas de β-caseínas, das κ-caseínas e α-caseínas, e estas estão ligadas por pontes de fosfato de cálcio. A extremidade das κ-caseínas forma uma camada “peluda” que mantém a repulsão eletrostática e impede a agregação adicional de submicelas. As micelas de caseína são estruturas não fixadas. Alterações de temperatura, pH, força, atividade da água, etc., levam a mudanças na distribuição do tamanho e na proporção de submicelas livres (além de mudanças no volume). A energia de interação bastante fraca entre submicelas evidencia a dinâmica do sistema. Outro aspecto dinâmico é a fusão de micelas de caseína ocorrendo depois de agregados, com o tempo ela passa de glóbulos para agregados.

Walstra (1999) conclui que as micelas de caseína estão sujeitas a reações de mudança, principalmente ao longo do processamento do leite. Além disso, o armazenamento do leite em tanques refrigeradores e coleta da matéria-prima a cada 48 horas aumentam as chances de multiplicação de bactérias psicrotróficas proteolíticas, que se desenvolvem em baixas temperaturas (0ºC a 15°C), produzindo enzimas termoestáveis que podem atuar sobre a κ-caseína, resultando na desestabilização do leite (Nornberg et al.; 2009).

O conteúdo de sais na forma solúvel e de cálcio iônico pode reduzir a estabilidade das caseínas do leite (Rose, 1961; Chaves et al, 2001), já que muitos minerais estão integrados nas micelas de caseína. A força iônica vinda de cátions como Na^+ e K^+ e o ânion Cl^- afeta diretamente a conformidade dielétrica da micela de caseína, podendo aumentar sua estabilidade (Horne & Parker, 1981). Entretanto, foi observado que o aumento do pH, relacionado com o aumento de teores de Na^+ e K^+, aumentou a estabilidade do leite (Horne & Muir, 1990).

A mobilidade de sais como Ca^{2+}, citratos e fosfatos afetam a estabilidade das micelas de caseína. Horne e Parker (1981), mostram o efeito da adição de Ca^{2+} na estabilidade do etanol do leite mantido em pH 6,5. Pequenas adições de ambas as espécies podem produzir grandes mudanças na estabilidade medida do etanol. O efeito da concentração do Ca altera o balanço iônico da micela de caseína, pois há uma redução das cargas negativas da micela de caseína, reduzindo a força de repulsão eletrostática entre elas, o que reduz a resistência da caseína para formar coágulos no teste de etanol ou calor (Barros et al., 1999).

Outro aspecto importante da estabilidade da micela de caseína está correlacionado à expressão dos genes polimórficos da β-lactoglobulina, e consequentemente, à estabilização da micela (Robitaille, 1995; Botaro et. al.; 2007). Há duas variantes no gene que expressa a β-lactoglobulina, a variante BB e AA (Botaro et. al., 2008).

2.7.2. Fatores ligados à alimentação/nutrição

A restrição alimentar reduz a estabilidade do leite de forma proporcional à magnitude e duração da restrição (Gabbi et al., 2016). Práticas de alimentação que acarretam ingestão de excesso de fibra reduzem a estabilidade do leite, como verificado por Barchiesi-Ferrari et al. (2007). Da mesma forma, o uso de volumosos de pior qualidade (menor digestibilidade e elevada fibra) reduziu a estabilidade do leite, na comparação de cana cortada e ensilada (Andrade et al., 2014). O fornecimento de dietas desbalanceadas em proteína e energia ou reduzidas em sua quantidade reduziu a estabilidade do leite (Zanela et al., 2006; Marques et al., 2010; Fruscalso et al., 2013; Stumpf et al., 2013; Schmidt, 2014). Um dos mecanismos sugeridos é o aumento da permeabilidade das junções firmes da glândula mamária, com aumento do influxo de sódio para o leite, aumento da concentração de cálcio iônico (Stumpf et al., 2013). O aumento de sódio em amostras de leite e do teor de lactose no sangue estão relacionados com menor estabilidade do leite e correlacionadas com a permeabilidade das junções (Chavez et al., 2004; Tsioulpas et al., 2007).

2.7.3. Distúrbios digestivos e metabólicos

Uma das principais respostas dos processos homeostáticos do corpo do animal frente a um distúrbio metabólico ou digestivo ocasionado, por exemplo, no período pós-parto com a entrada do animal em uma situação de BEN é a acidose. A acidificação sanguínea (Marques et al., 2011; Fagnani et al., 2014) ou ruminal (Werncke, 2017) foi associada à menor estabilidade do leite. Um dos possíveis mecanismos é o aumento da concentração de cálcio iônico (Marques et al., 2011; Martins et al., 2015; Werncke, 2017).

2.7.4. Estádio de lactação

Os menores valores de estabilidade são encontrados usualmente nos primeiros dias de lactação, uma vez que estudos mostram valores de estabilidade inferiores a 74% durante as primeiras 2 a 3 semanas e novamente inferiores em no final da lactação, ainda que exista variação muito grande durante a lactação (Tsioulpas et al., 2007; Marques et al., 2010). Esse efeito pode ser em parte explicado pelas modificações na integridade das junções firmes nas células epiteliais da glândula mamária (Neville, 1995; Stumpf et. al., 2013). Com o avanço da lactação, verificou-se um decréscimo dos teores de fósforo e cálcio solúvel e manutenção do teor de cálcio iônico e com isso, redução da estabilidade térmica, mas com grandes variações com o avanço da lactação (Barros et al., 2002; Rose 1961).

2.7.5. Estresse térmico

O estresse térmico pode reduzir a estabilidade do leite de forma proporcional à magnitude e duração da exposição às altas temperaturas e/ou radiação solar (Abreu et al., 2011). Os efeitos do estresse térmico sobre a estabilidade do leite no teste do álcool podem ser relacionados à redução no consumo de alimentos dos animais(Zanela et al., 2006; Abreu et al., 2011; Stumpf et al., 2013). O mecanismo não está definido e pode estar relacionado ao aumento da acidez, observado em situação de estresse térmico severo (Abreu, 2015; Abreu et al., 2020).

Segundo Sevi & Caroprese (2012), exposição ao Sol em altas temperaturas pode promover aumento na permeabilidade dos capilares, elevando a quantidade de enzimas proteolíticas no leite, de forma a reduzir as concentrações proteicas do mesmo. Em adição, a redução na oferta e consumo de forrageiras nos pastos nos meses mais quentes, bem como o menor aporte de energia e nitrogênio, contribuem para os achados. A principal causa da redução nos teores de proteína em estudo conduzido por Bernabucci et al. (2002) foi a redução nos níveis de α e β-caseínas, provavelmente em virtude do menor aporte de energia e proteína. Essas caseínas são ricas em grupos fosfato e são os componentes das micelas (Schmidt, 1980).

2.7.6. Composição do leite estável e do LINA

Existe grande variabilidade quanto aos resultados da composição química do leite normal comparado com o LINA. O teor de gordura foi superior para o LINA comparada com o leite normal (Barros, 2001; Marques et al., 2007). Barros (2001) observou maior concentração de proteína do leite positivo ao teste do álcool. Entretanto, Lopez (2008) e Marquez et al. (2004) encontraram valores inferiores de proteína para o LINA, quando comparado com o leite normal.

2.7.7. Temperatura e duração da armazenagem do leite

Falhas na temperatura de conservação do leite, contagens iniciais elevadas de microrganismos resultam em expressiva multiplicação bacteriana. Segundo Roberts (1979) e Hamman (1979), a temperatura de armazenamento do leite deve ser menor do que 4°C para armazenamentos superiores a 24 horas. Henzl et al. (2015) verificaram que a temperatura do leite transportado aumentou com o incremento da temperatura do ar externo, mas os teores de proteína e gordura do leite diminuíram. Os microrganismos psicrotróficos, consequentes da falta de higiene e de elevadas contagens padrão em placas (CPP), produzem enzimas como as proteases e lipases, que provocam sérios problemas tecnológicos e alterações sensoriais no leite (Yamazi et al., 2013).

2.7.8. Fatores relacionados à agitação do leite

O enfoque principal nos estudos que avaliam o efeito do transporte é a temperatura e não a agitação, que é uma variável que tem impacto, principalmente em relação à estrutura das caseínas, contudo não foi muito elucidada. Todavia, na

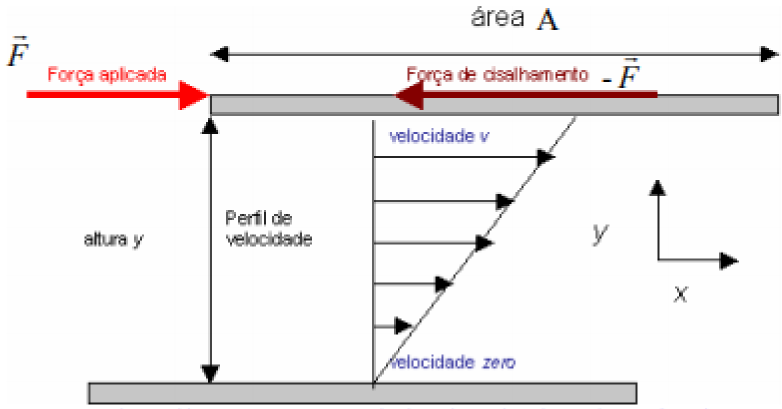
Alemanha levantou-se a hipótese sobre o tamanho das rotas de recolhimento de leite e Müller (2006) discorre sobre a modelagem das rotas que afeta diretamente o tempo em que o leite vai ser submetido à agitação até a chegada à indústria. Ele concluiu que pouquíssimos trabalhos avaliam ou criam ferramentas que possam melhorar a modelagem de rotas de recolhimento, de modo estratégico e com menor gasto de combustíveis. Em condições laboratpriais, a agitação reduziu a estabilidade do leite provavelmente pelo aumento do cálcio iônico e da acidez observados (Warmiñska et al., 2006). Além disso, a agitação induz a lipólise, ao aumentar a entrada de ar no leite, incrementando a velocidade de reação da lipólise e reduzindo os teores de gordura do leite (Deeth & Fitzgerald, 1976).

Outros estudos sobre o efeito da agitação nas características físico-químicas do leite mostraram que, mesmo se as condições térmicas e sanitárias durante o transporte forem mantidas, interações mecânicas, principalmente choques e vibrações, influenciam negativamente a estrutura física de determinados componentes e na qualidade final da matéria-prima (Palich, 1993; Warmiñska et al., 2003). Warmiñska & Kruk (2001) e Warmiñska et al. (2003) mencionam que o transporte do leite submete o líquido a agitações verticais e horizontais de alta frequência diminuindo a estabilidade do leite ao teste do álcool. Além disso, os a lipólise, proteólise e a acidez foram aumentadas durante o armazenamento do leite cru sujeito à vibração (Czerniewicz et al., 2006). Warmiñska et al. (2006) relacionaram a menor estabilidade do leite submetido à maior frequência de agitação e à acidez aumentada. Os autores atribuíram os seus resultados ao efeito mecânico de ruptura da estrutura micelar, reduzindo a hidratação das micelas e expondo os grupos negativos para fora da micela, o que aumenta a acidez e a concentração de Ca^{2+}.

Nesse sentido, faltam dados que relacionem as características das rotas de coleta de leite (condições da estrada em termos de pavimentação e duração da viagem) sobre a agitação do leite. Ainda que um número considerável de estudos aborde a agitação e seu efeito nas características físico-químicas do leite, nenhum deles explica em termos físicos como a agitação pode influenciar de forma negativa a conformação das moléculas do leite, e assim alterar as características físico-químicas. A compreensão do fenômeno físico acontece com o leite no compartimento do caminhão de coleta a granel que o leva até a indústria para o processamento pode contribuir para compreender e predizer a qualidade do leite entregue nas plataformas de recepção das indústrias.

Os caminhões tanque que transportam o leite ficam sujeitos ao fenômeno chamado "*slosh*". De acordo com Liebert (2009) "o efeito *slosh* em recipientes parcialmente cheios de líquido é motivo de preocupação para engenheiros, pois líquidos em recipientes de formatos arbitrários, sob excitações externas, resultam em turbulência. A natureza de tal turbulência é bastante complexa devido a diversos efeitos, como o gradiente de pressão, por exemplo. Dependendo do tipo de perturbação e forma do recipiente, a superfície livre do líquido pode experimentar diferentes tipos de movimento, como planar simples, não plana, rotativa, batimento irregular, simétrico, assimétrico, quase-periódico e caótico". A amplitude do efeito "*slosh*" depende da amplitude e frequência do movimento do tanque, do tipo de líquido de enchimento, da profundidade de enchimento, das propriedades de líquidos e da geometria do tanque. O tanque do caminhão transportador de leite é do tipo policêntrico. Nesses tipos de tanque, o movimento é mais complexo e o centro de gravidade da carga se desloca primeiramente na horizontal e depois descreve um movimento elíptico, o que pode aumentar o risco de tombamento e também exercer maior turbulência dentro do líquido.

Para entender melhor esse fenômeno devemos compreender alguns conceitos de mecânica de fluidos hidrodinâmicos (sujeitos ao movimento). Cattani (2005) fala sobre o comportamento dos fluidos reais. Ao analisar a situação em que o leite se encontra confinado em um compartimento, admite-se que ele se torna um fluido real, isso porque há uma dissipação de energia mecânica devido a atritos internos (causado pela viscosidade do leite - coesão entre as moléculas) entre as partículas do fluido ou do fluido com o ambiente. Sabendo que o leite é um fluido que possui viscosidade e resistência à deformação pela conformidade estável de seus constituintes. No caso, a deformação se refere às forças de cisalhamento que o leite sofre ao ser agitado (turbulência) ao longo do processo de transporte, como mostra Figura 3.



Fonte: http://www.setor1.com.br/analises/reologia/cisa_figu.htm

Figura 3. Força de cisalhamento aplicada sobre um fluido.

O leite é classificado como fluido newtoniano, uma vez que a viscosidade não vai alterar independente da força de cisalhamento que ele sofre. Essa situação pode ser demonstrada pela equação:

$$\tau_{yx} = -\mu \frac{du_x}{dy}$$

Na qual:

τ_{yx} é a tensão de cisalhamento na direção x, g/cm.s^2;

$\frac{du_x}{dy}$ é o gradiente de velocidade ou taxa de cisalhamento, s^{-1};

μ é a viscosidade, cP = 10^{-2}g/cm.s = 0,001kg/m.s = 10^{-3} N.s

Ou seja, quanto maior a taxa de cisalhamento, maior é a taxa de deformação do fluido. No caso do leite dentro do tanque, durante o transporte, quanto maior a força de cisalhamento maior será a deformação do fluido. Desta forma, o efeito da agitação pode ser explicado fisicamente, demonstrando os efeitos negativos sobre as características físicas do leite, como a estabilidade das micelas de caseína, uma vez que vai atuar na deformação de suas moléculas, mais especificamente no desdobramento de moléculas de proteínas, mencionada por Warmiñska & Kruk (2001), e consequentemente na estabilidade do leite, dependendo da magnitude da frequência.

Uma das hipóteses levantadas é que ao agitar o leite dentro do tanque do caminhão de transporte há uma geração de energia pelo atrito entre as partículas de leite. Essa energia, por sua vez, se transforma em calor, e este afeta a estabilidade da caseína. O'Connell et al. (2006) concluíram que altas temperaturas afetam a quantidade de fosfato de cálcio associado às micelas, o que aumenta a dissociação da κ-caseína, diminuindo a estabilidade do leite.

Após realizar esta retrospectiva do que a literatura nos apresenta sobre estabilidade do leite e LINA, verifica-se a sua complexidade e os vários fatores que afetam a estabilidade das caseínas do leite. Com isso, espera-se, no presente estudo, apresentar uma síntese sobre os fatores que afetam a estabilidade do leite cru bovino através da revisão sistemática e posterior meta-análise dos dados, conectando os diversos fatores, desde os animais e os não ligados ao animal, principalmente os relacionados ao transporte a agitação, e se de fato, a agitação durante o transporte da unidade produtiva até a indústria pode diminuir sua estabilidade.

3. HIPÓTESES E OBJETIVOS

3.1 Hipótese

A estabilidade do leite é uma propriedade que pode ser afetada por múltiplos fatores, incluindo fatores ambientais como a estação do ano, agitação, higiene, entre outros; e fatores ligados ao animal como restrição alimentar, estágio de lactação, frações da caseína, entre outros.

3.2 Objetivos

Objetivos Primários

- O objetivo é determinar os principais fatores que afetam a estabilidade do leite (revisão sistemática) e calcular o efeito médio dos principais fatores, como a estação do ano e a restrição alimentar (meta-análise).

Objetivos Secundários

- Avaliar sistematicamente os fatores que afetam a estabilidade do leite cru bovino e elencar as lacunas nesta área de pesquisa.

CAPÍTULO II

Factors affecting the thermal stability of raw bovine milk: a systematic review

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Abstract

Milk components such as its protein and mineral fractions play a fundamental role in the ethanol-stability of milk, maintaining its characteristics during its processing. Therefore, the objective of this study was to elaborate a systematic review on the factors that affect the thermal stability of raw bovine milk, tested by alcohol and coagulation time tests. A search key was elaborated through the acronym Pico, where it was sought to filter articles that addressed the factors that affect the thermal stability of raw bovine milk. After the evaluation of the abstracts, 87 original papers were selected: 64 evaluated milk stability with the alcohol test, 18 used the coagulation time test and 4 used both tests. After analyzing the full texts, 35 factors related to the thermal stability of milk were identified and descriptive statistical analysis was applied. The publications covered a period from 1923 to 2021, countries with the highest number of publications were Brazil with 32 articles, followed by Ireland with 11. The factors most frequently studied were season, diet restriction and milk composition. Ethanolic concentration in solution tests varied between studies, the most used was 68°GL (21.3%), followed by 70 and 72to76° degrees GL (14.7%). Feed restriction, heat stress, illnesses, extreme lactation periods decreased milk stability. In the normal pH range of milk, alcohol test and coagulation time present very low association. The stability of milk in the alcohol test is influenced by several factors, and despite its lack of specificity, there is no other rapid test for its replacement. Its use remains in countries where raw milk quality is very variable and/or challenging thermal processing such as UHT is employed.

Key Words: milk stability, non-acid, unstable milk, raw milk, bovine milk

Introduction

Thermal stability of raw milk, mentioned in this work as stability of milk, has been studied due to the need for milk to present resistance to heat processing at the dairy industry, especially during UHT milk, powdered milk, evaporated or condensed milk. Milk components, such as proteins and minerals, play a key role in the thermal stability of milk, maintaining its characteristics during processing. Caseins are the main milk proteins, comprising about 80% of the total proteins, and are present in four main forms: α1-, α2-, β- and κ-casein (Fox & Brodkorb, 2008), which have high thermal stability, suffering little or no effect during pasteurization. Casein supports heating to 100°C for 24 hours at normal pH (6.7) without coagulation (O'Connell & Fox, 2000; Singh, 2004), which allows dairy industries to perform milk treatment at elevated temperatures such as Ultra High Temperature Milk (UHT). According to Singh & Creamer (1992), protein fractions, β-lactoglobulin (LG) and k-casein (κ), have a major influence on milk stability.

Milk protein stability can be defined as the ability of casein to remain in colloidal suspension and lactalbumin and lactoglobulin to remain in solution when milk is subjected to heat or other modifying influences (White & Davies, 1958). There are several methods for evaluating milk stability, such as alcohol or ethanol test (White & Davies, 1958; Chaves et al. 2004), heat coagulation time test of milk (White & Davies, 1958, Gulati et al. 2019; Abreu et al. 2020), phosphate test (Ramsdell, Johnson & Evans 1930) and rennet gelatinization (Lin et al. 2009), although there are doubts about how much they estimate milk stability during the industrial process (Singh, 2004). This systematic review will address alcohol and heat coagulation time tests as estimators of thermal stability, as they are the most commonly used in industry and research, respectively.

The alcohol test evaluates the stability of milk proteins submitted to dehydration caused by alcohol (Omoarukhe et al., 2010). The addition of ethanol to milk induces several changes in casein micelles, such as the collapse of the κ-casein layer, the reduction in micellar loading, and the precipitation of calcium phosphate, which contribute to reduce the micellar stability of κ-casein, with consequent coagulation (O'Connell et al., 2006). The alcohol test (ethanol or alizarol, when a pH indicator, alizarin, is added) is a method widely used in industries in various countries such as Brazil (Brasil, 2018), Argentina, Uruguay, Russia, Cuba, Taiwan, India and

South Africa (Machado et al., 2017; Martins et al., 2015). However, it is no longer used in countries such as Australia, Canada, the United States, New Zealand and others. The stability of milk to ethanol (Milk Ethanol Stability - MES) was defined as the minimum concentration of added aqueous ethanol capable of inducing milk coagulation (Horne & Parker, 1980).

According to Dumpler, Huppertz & Kulozik (2020), the heat coagulation time test, which dates back to its use in the early 20th century (Sommer & Hart, 1919), was related to the heat stability of milk, historically defined as the time necessary to visually coagulate a milk sample at constant heating temperature in oil bath for laboratory evaluation scale. The test of the coagulation time (TCT) to the heat is not used at the industrial level, only in researches that address the present theme.

Both tests are the most mentioned in the literature, even though the methodology and many of the factors that affect them are distinct. The choice between the two tests is often due to the ease of the MES compared to the heat coagulation time test. The correlation between MES and TCT is low (Molina et al., 2001; Negri, 2002; Chavez et al., 2004).

It is necessary to recognize, however, that the low stability of milk can effectively impair industrial processes that require high thermal resistance of milk, such as UHT milk, milk powder (Ramsdell, Johnson & Evans, 1931; Tsioulpas, Lewis & Grandison 2007) and milk-based alcoholic beverages (Donnelly & Horne 1986). Moreover, there is a lack of studies that show the limit of thermal stability of milk needed for each of the different milk processes (Fischer et al., 2012). In this sense, the objective of this study was to elaborate a systematic review on the factors that affect the thermal stability of raw bovine milk, evaluated by the alcohol test or by the test of coagulation time to heat.

Material and methods

The proposed theme is the stability of raw bovine milk. The main objective is to carry out a systematic review of the world literature on the main factors affecting the thermal stability of milk, using scientific studies, elaborating a historical panorama of the thermal stability of milk, by checking the alcoholic grade most used in the alcohol test, as it is still the most used test to verify the stability of raw bovine milk.

The PRISMA methodology was used. The question of the review elaborated after the definition of the research objective consisted in evaluating which factors affect the thermal stability of raw milk, using the alcohol test, or heat coagulation time/temperature. The search key included, through the acronym Pico, the objectives and the question of the review, where P refers to POPULATION (population) - raw bovine milk, which may include samples of individual animals and/or bulk milk, on the farm; INTERVENTION (intervention/interest) - related to the thermal stability of milk; CONTEXT (context) - factors that can affect the thermal stability of milk. The search key consisted of the terms: "("milk") AND ("LINA" OR "unstable" OR "Stability" OR "Ethanol" OR "Alcohol" OR "non-Acid" OR "Heat") AND ("feed Restriction" OR "Thermal stress" OR "Heat stress" OR "Lactation Stage" OR "Metabolic disease" OR "Metabolic disturb" OR "Digestive disease" OR acidosis OR mastitis OR agitation OR Storage OR transport)". The search was carried out on July 17, 2020, in three databases: Pubmed, Scopus and Web of Science. Filters were not used in the databases. All records were selected and stored in the Mendeley desktop software for analysis. The information contained in the results were title, authors, year, publication vehicle, DOI, Abstract and keywords.

Search methods and selection criteria

With the *a priori* withdrawal of duplicate articles, the eligibility of the articles was checked, and the summaries were evaluated independently by three independent observers. Abstracts were selected for subsequent inspection if they met the criteria:

A) related to scientific articles and/or short papers published in journals;
B) access to the full text;
C) use of raw bovine milk, individual or tank milk;
D) contain the word "stability" or equivalent related to raw milk;
E) included alcohol test and alcohol graduations or thermal stability by heating and clotting time;

Searches in the three databases totaled 5,466 results, 2,692 were excluded as duplicate results, leaving 2,774 articles evaluated by three observers, which reached 30 articles. After this stage, a subject specialist was asked to verify the existence of other unfiltered papers by the search key. Twenty-seven articles were identified and inserted, totaling 57. These 57 articles were analyzed and four of them did not meet the selection criteria. Thus, 53 articles were eligible and their bibliographic references were inspected to ensure that no articles related to the subject were left out of the selection by the search key. Thus, from the references of articles 53, we found 31 more articles that fit the same criteria described above, totaling 84 articles to be evaluated completely.

A second search was carried out, with the same key, in the three databases on October 27, 2020, after the end of the selection of articles and bibliographic references of the same, to verify if any other article that could contribute to the systematic review had been published. We found three more articles published two months after the first search, which met the selection and eligibility criteria, reaching a final number of 87 papers. The search and selection process uses the PRISMA flowchart (Figure 1).

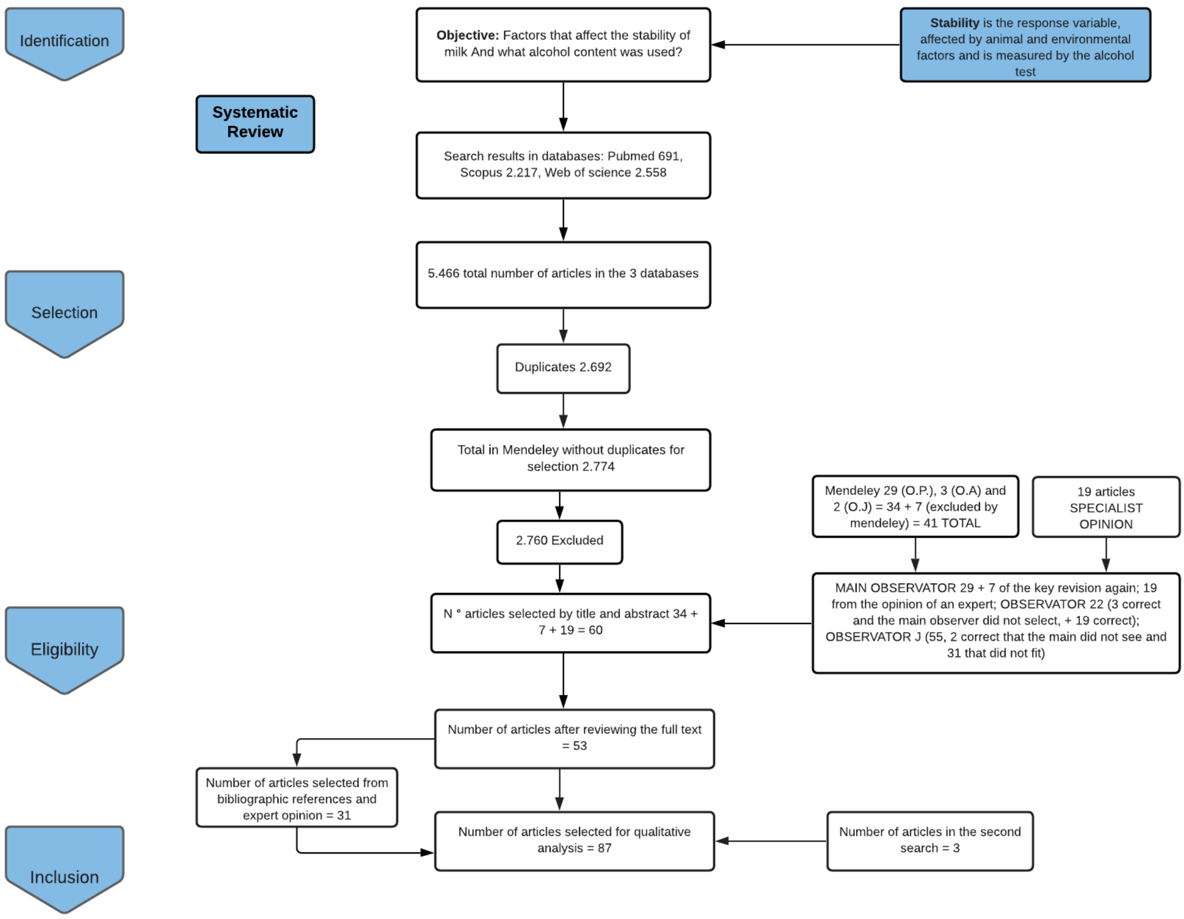

Figure 1. PRISMA flowchart

Data processing and analysis

Statistical analyses were performed using R-studio (1.3.959-1, "Middlemist Red") software. The Shapiro-Wilk test, proposed in 1965 (Shapiro & Wilk, 1965), based on the W statistic given by the equation \[W=\frac{b^2 {\displaystyle\sum_{i=1}^n (x_{(i)}-\bar{x})^2}\]. Data did follow normal distribution and descriptive statistical analysis was performed using data on frequency, probability, median, fashion and quartiles.

Results

Factors affecting milk stability

The analysis of the complete articles revealed that 35 factors were related to milk stability (Table 1). The most frequent factors affecting milk stability were the season, feed restriction and milk composition (Table 1), being evaluated in 9.5, 7.9 and 7.1% of the articles, respectively. Season and feed restriction factors are related to the quantity (Stumpf et al., 2016; Hernandez & Ponce 2005) and composition (quality) of food available to animals (Martins et al., 2019; Gabbi et al., 2018; Machado et al., 2014; Marques et al., 2010), that consequently, also affects milk composition (Fagnani et al., 2017; Rathnayake et al., 2016; Oliveira & Timm 2006; Chavez et al., 2004). Also, season might be related to the heat stress, diminishing MES (Abreu et al., 2021).

Table 1. Frequencies of factors affecting milk stability

Factor	Frequency	Percent (%)
Season	11	9,5
Restriction on feeding animals	10	7,9
Effect of milk composition	9	7,1
pH and stability ratio	8	6,3
Mastitis	7	5,5
Lactation stage	7	5,5
Genetic polymorphism of animals	6	4,7
Ionic calcium in milk	6	4,7
Variation of salts in milk	5	3,9

SCC	5	3,9
Milk quality	5	3,9
Occurrence of LINA	4	3,1
Milk agitation	4	3,1
Storage	3	2,3
The ownership structure	3	2,3
Method of analysis for milk	3	2,3
Supplementary feeding	3	2,3
Protein levels and/or diet energy	3	2,3
Food degradability of the diet	2	1,5
Temperature	2	1,5
Anionic-cation diet	2	1,5
Thermal stress	2	1,5
Thermal treatments in milk	2	1,5
Type milk A and B	2	1,5
Dietary zinc	1	0,7
Metabolic disorders	1	0,7
Lactoperoxidase	1	0,7
Animal management	1	0,7
Breed of cows	1	0,7
Transglutaminase-induced crosslinking (TGase)	1	0,7
Level of milk production	1	0,7
Blood composition of cows	1	0,7
Whey salts and proteins	1	0,7
Removal of milk fat	1	0,7
Relationship with alcohol testing	1	0,7

The records of selected manuscripts, with the respective title, author and year of the publication and type of test to measure milk stability (Table 2).

Table 2. Search key results and review of bibliographic references

Title	Author and Year	Test
Occurrence of Unstable non-acid milk (UNAM) on commercial farms in the extreme west of Santa Catarina	Manske et al., 2020	Alcohol

Natural tree shade increases milk stability of lactating dairy cows during the summer in the subtropics	Abreu et al., 2020	Alcohol e heat
Seasonality and collection routes influence the occurrence of non-acid unstable milk, the density and cryoscopy of milk supplied to a dairy industry in northern Minas Gerais	Oliveira et al., 2020	Alcohol
Ultrasound stabilization of raw milk: Microbial and enzymatic inactivation, physicochemical properties and kinetic stability	Scudino et al., 2020	Ultrassom
Effect of reducing daily herbage allowance during early lactation on composition and processing characteristics of milk from spring-calved herds	Gulati et al., 2019	Heat
Natural variations of citrate and calcium in milk and their effects on milk processing properties	Akkerman et al., 2019	Alcohol
Effect of dietary crude protein degradability and corn processing on lactation performance, milk protein composition, and stability	Martins et al., 2019	Alcohol
Subclinical intramammary infection does not affect bovine milk ethanol stability	Martins et al., 2019	Alcohol
Dietary Zinc-Amino Acid Complex Does Not Affect Markers of Mammary Epithelial Integrity or Heat Stability of Milk in Mid-Lactating Cows.	Shafer et al., 2018	Heat
Assessment of Raw Cow Milk Quality in Smallholder Dairy Farms in Pemba Island Zanzibar, Tanzania	Gwandu et al., 2018	Alcohol
Different levels of supplied energy for lactating cows affect physicochemical attributes of milk	Gabbi et al. 2018	Alcohol
Effect of agitation, gathering place and cooling time on milk quality of Jersey cows	Angelo et al., 2018	Alcohol and heat
Seasonal variation in the composition and processing characteristics of herd milk with varying proportions of milk from spring-calving and autumn-calving cows	Lin et al., 2017	Alcohol
The effect of housing system of simmental cows on processing suitability of milk and quality of dairy products	Radkowska et al., 2017	Alcohol
Seasonal variation, method of determination of bovine milk stability, and its relation with physical, chemical, and sanitary characteristics of raw milk	Machado et al., 2017	Alcohol

Field findings about milk ethanol stability:a first report of interrelationship between α-lactalbumin and lactose	Fagnani et al., 2017	Alcohol
The effect of storage conditions on the composition and functional properties of blended bulk tank milk	O'Connel et a., 2017	Heat
Changes in Compositional and Keeping Quality Parameters of Cow Milk on Ethanol Stability	Rathnayake et al., 2016	Alcohol
Behaviors associated with cows more prone to produce milk with reduced stability to ethanol test due to feeding restriction	Stumpf et al., 2016	Alcohol
Milk quality and profile of dairy farms in southern Santa Catarina: Multivariate approach	Werncke et al., 2016	Alcohol
Alcohol Stability of Milk from the Perspective of X-Ray Diffractometry	Fagnani et al., 2016	Alcohol
Milk traits of lactating cows submitted to feed restriction	Gabbi et al., 2015	Alcohol
Effect of dietary cation-anion difference on performance of lactating dairy cows and stability of milk proteins	Martins et al., 2015	Alcohol
Concentrate: forage ratio in the diet of dairy cows does not alter milk physical attributes	Machado et al., 2014	Alcohol and heat
Effect of milk sample temperature on the concentration of soluble calcium and beta-casein: Interference with the ethanol stability test	Costa et al., 2014	Alcohol
Influence of production season and lactation stage on the technological suitability of milk from cows of various breeds fed in the TMR system	Barlowska et al., 2014	Heat
Effect of seasonal variation on the composition and properties of raw milk destined for processing in the UK	Chen et al., 2014	Alcohol
Acid-base balance of dairy cows and its relationship with alcoholic stability and mineral composition of milk	Fagnani et al., 2014	Alcohol
Microbiology and physical chemical characterization of unstable non-acid milk according to the seasons	Battaglini et al., 2013	Alcohol
Feeding restriction impairs milk yield and physicochemical properties rendering it less suitable for sale	Fruscalso et al., 2013	Alcohol

Severe feed restriction increases permeability of mammary gland cell tight junctions and reduces ethanol stability of milk	Stumpf et al., 2013	Álcool
Evaluation of lactoperoxidase system as raw milk preservative at different storage temperature conditions in the central highlands of Ethiopia	Kassa et al., 2013	Alcohol
Quality of raw grade B milk from four farms in the region of vale do taquari, RS, Brazil	Fava et al., 2012	Alcohol
Estimation of correlation between somatic cell count and coagulation score of bovine milk	Atasever et al., 2012	Alcohol
Electrophoretic characterization of proteins and milk stability of cows submitted to feeding restriction	Barbosa et al., 2012	Alcohol
Milk yield, milk composition and biochemical blood profile of lactating cows supplemented with anionic salt	Marques et al., 2011	Alcohol
Effects of different calcium salts on properties of milk related to heat stability	Omo et al., 2010	Alcohol
Effects of stabilizer addition and in-container sterilization on selected properties of milk related to casein micelle stability	Tsioulpas et al., 2010	Alcohol
Supplementation of Holstein cows at an extended lactation stage	Marques et al., 2010	Alcohol
Supply of supplements with different energy and protein levels to Jersey cows and their effects on milk instability	Marques et al., 2010	Alcohol
Effect of the Lactation Period and Milk Stability on Physical-Chemical Characteristics	Barbosa et al., 2009	Alcohol
Study on cow’s raw milk quality in the eastern Taiwan during hot season	Lin et al., 2009	Alcohol
Prevalence of clinical and subclinical mastitis and quality of milk on smallholder dairy farms in Tanzania	Mdegela et al., 2009	Alcohol
Effect of the kappa-casein gene polymorphism, breed and seasonality on physicochemical characteristics, composition and stability of bovine milk	Botaro et al., 2009	Alcohol
Investigation of the effects of season, milkingregion, sterilization process and storage conditions on milk and UHT milk physico-chemical characteristics: a multidimensional statistical approach	Gaucher et al., 2008	Alcohol And heat

Effect of Minerals on Casein Micelle Stability of Cows' Milk	Tsioulpas et al., 2007	Alcohol
Inestabilidad de la leche asociada a componentes lácteos y estacionalidad en vacas a pastoreo	Barchiesi et al., 2007	Alcohol
Influence of composite κ-casein and β-lactoglobulin genotypes on composition, rennetability and heat stability of milk of cows of Slovak Pied breed	Michalcovál & Krupová, 2007	Alcohol
Occurence of unstable to alcohol 76% and non-acid milk (LINA) and influence on physico-chemical aspects of milk	Marques et al., 2007	Alcohol
Ethanol stability of casein micelles cross-linked with transglutaminase	Huppertz & Kruif, 2007	Alcohol
Influences of Different Milk Yields of Holstein Cows on Milk Quality Indicators in the Czech Republic	Janu et al., 2007	Alcohol
Effect of mastitis on raw milk compositional quality	Ogola et al., 2007	Alcohol
Effect of vibration frequency and exposure time on the technological usability of fresh milk	Warminska et al., 2006	Alcohol
Storage stability of raw milk subjected of vibration	Czerniewicz et al., 2006	Alcohol
Unstable nonacid milk and milk composition of Jersey cows on feed restriction	Zanela et al., 2006	Alcohol
Composition of milk with casein instability	Oliveira & Timm, 2006	Alcohol
Effect of three types of diet on the appearance of metabolic disorders and their relationship with alterations in the composition of milk in Holstein Friesian cows	Hernandéz & Ponce, 2005	Alcohol
Bovine milk composition parameters affecting the ethanol stability	Chavez et al., 2004	Alcohol
Heat and ethanol stabilities of high-pressure-treated bovine milk	Huppertz et al., 2004	Alcohol
Ratio of the 72% (v / v) Alizarol test in "fresh" cow's milk with acidity and somatic cell count: microbiological analysis	Donatella, 2003	Alcohol
Correlation between thermal stability and proof of alcohol milk at the level of a dairy collection center	Molina et al., 2001	Alcohol

Effect of a differential allelic expression of kappa-casein gene on ethanol stability of bovine milk	Robitaille et al., 2001	Alcohol
The Two-Stage Coagulation of Milk Proteins in the Minimum of the Heat Coagulation Time-pH Profile of Milk: Effect of Casein Micelle Size	O'Connell & Fox, 2000	Alcohol
Influence of kappa-casein and beta-lactoglobulin phenotype on the heat stability of milk	Paterson et al., 1999	Heat
Alcohol stability of milk and its relation to milk and blood composition in Holstein dairy cows	Sobhani et al., 1998	Alcohol
Influence of kappa-casein and beta-lactoglobulin genetic variants on the heat stability of milk	Robitaille et al., 1995	Heat
Effect of genetic-polymorphism on the thermal-stability of beta-lactoglobulin and kappa-casein mixture	Imafidon & No-Kwachang, 1991	Heat
Heat stability of milk: influence of colloidal and soluble salts and protein modification on the pH-dependent dissociation of micellar K-casein	Sing & Fox, 1987	Heat
Factors affecting the ethanol stability of bovine skim milk: VII. Lactational and compositional effects	Horne, 1986	Alcohol
Relationship between ethanol stability of bovine milk and natural variations in milk composition	Donnelly & Horne, 1986	Alcohol
Factors affecting the ethanol stability of bovine milk.: I. Effect of serum phase components	Horne & Parker, 1981	Alcohol
Heat stability of milk: interrelationship between assay temperature, pH and agitation	Hyslop & Fox, 1981	Heat
Studies on the heat stability of milk protein II. Effect of exposing milk to light.	Sweetsur & White, 1975	Heat
Studies on the heat stability of milk protein I. Interconversion of type A and type B milk heat-stability curves	Sweetsur & White, 1974	Heat
Effects of subclinical mastitis on heat stability of fluid milk	Feagan et al., 1966	Heat
The stability of milk protein to heat II. Effect on heat stability of aging milk at different temperatures	Davies & White, 1966	Heat
Factors affecting the heat stability of milk	Rose, 1962	Heat
Variations in the Heat Stability and Composition of Milk from Individual Cows during Lactation	Rose, 1961	Heat

The relation between the chemical composition of milk and the stability of the caseinate complex: II. Coagulation by ethanol	Davies & White, 1958	Alcohol
The relation between the chemical composition of milk and the stability of the caseinate complex. I. General introduction, description of samples methods and chemical composition of samples	White & Davies, 1958	Alcohol
The heat coagulation of milk	Pyne & McHenry, 1955	Heat
Milk instability following an increase calcium ion content	Seekles & Smeets, 1952	Alcohol
Studies on the alcohol coagulation of fresh cow milk	Mitamura, 1937	Alcohol
The heat coagulation of milk. 1. Variations in compositions, heat stability, and others tests of milks from four cows during the course of a lactation period	Holm, 1932	Heat
A test for detection of milk unstable to heat	Ramsdell, 1931	Heat
Studies on the stability of evaporated milk during sterilization, with reference to the hydrogen ion concentration, alcohol test and the addition of specific buffers	Benton & Albery, 1926	Alcohol
Study of the factors that influence the coagulation of milk in the alcohol test I	Sommer & Binner, 1923	Alcohol

History of publications on milk stability

Based on the 87 selected articles, a graphic (Figure 2) was drawn with the historical distribution of the publications. The first article shown in the timeline was published by Sommer and Binney (1923). Chronological distribution of publications about MES is odd, highly concentrated in the last 20 years. It is worth to notice that after 100 years, in 2020, and other publications most recent (Hanuš et al., 2021; Martins et al., 2021) showed the relevance of stability assessment, since low stability can compromise the thermal process of milk sterilization, increasing sediment and gelification, reducing the efficiency of the industrial process (Lin et al. 2017), as UHT milk plays a major role as an international commodity, exerting economic impact on producers and industries.

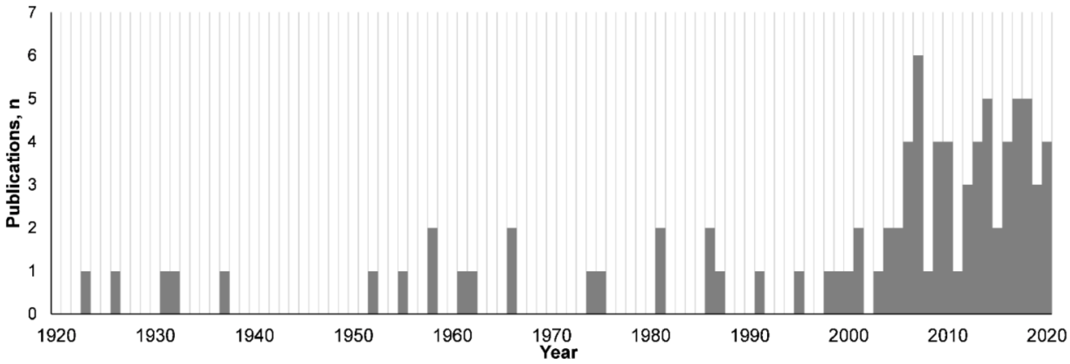

Figure 2. Historical distribution of publications on the stability of raw bovine milk.

Worldwide behavior of publications on milk stability

Countries with more published studies were Brazil (32 articles), followed by Ireland with 11, United Kingdom with 6, United States, Scotland and Canada with 5, Poland with 4, Chile and France with 2, the rest with only 1 publication (Figure 3). Of the 25 countries that studied milk stability, Brazil and Ireland stood out, with results on the regional occurrence (Oliveira et al., 2007, Zanela et al., 2009, Marques et al., 2007, Machado et al., 2010; Battaglini et al., 2013; Manske et al., 2020; Oliveira et al., 2020) as well about factors affecting milk stability (O'Connel 2017; Huppertz & Kruif 2007; Sing & Fox 1987; Pyne & Mchenry 1955), indicating that this problem can be recurrent, causing problems in the feasibility of using and industrializing raw milk.

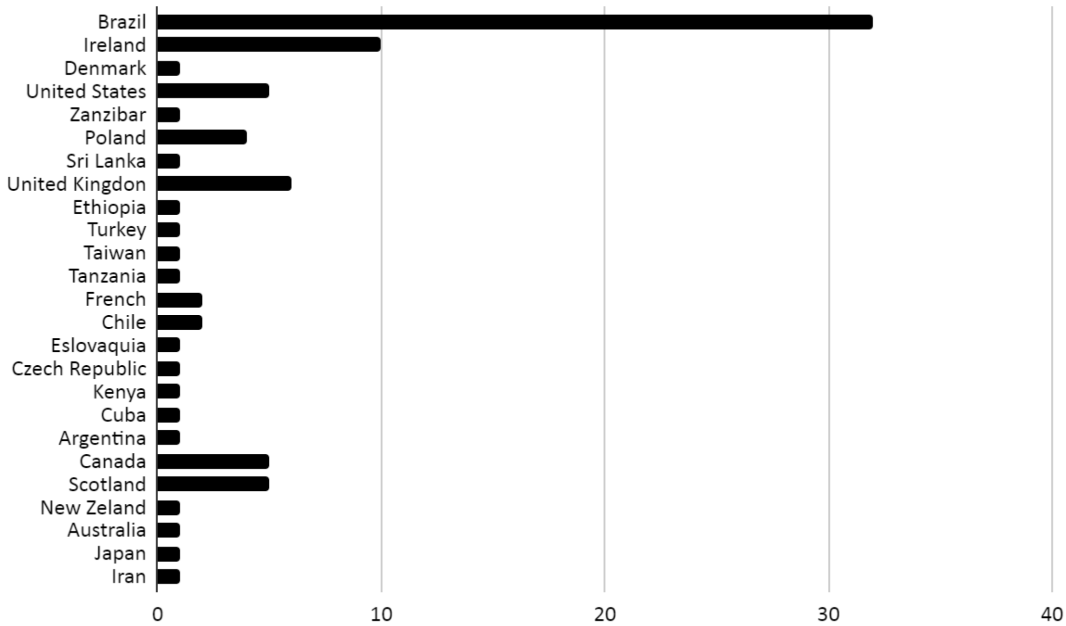

Figure 3. Number of publications on the stability of raw bovine milk by nationality

Relation of the alcohol test with the alcoholic grades used

Historically, the alcohol grade used in the test has been altered. Early publications, dated to the early 20th century, tested alcoholic degrees between 70 and 75°GL (Sommer & Binner, 1923; Benton & Albery, 1926). In the following decade, the study by Mitamura (1937) compared several alcoholic degrees, from 66° to 94°GL, to test the most suitable for performing the test, and reported that milk samples coagulated at 78 to 80°GL.

Molina et al. (2001) conducted a study to determine the correlation between ethanol concentration in the test solutions and the thermal stability of the milk (heat coagulation time), using ethanol concentrations of 70, 75, 80 and 85°GL. These authors observed that at 75°GL, values of thermal stability of 60 to 70 seconds at 135 °C were obtained. This time was considered sufficient to produce UHT milk (135 °C to 140 °C for 2 to 4 seconds).

In Brazil, the ethanol concentration used until the 90s was 68ºGL, raising to 70 during the 90s and, then in the early 2000s it raised to 76. More recently dairy industries raised it to 78, 80 and even 82°GL. Differences in ethanol concentration (°GL) in the solution test occur until the present between the countries, being 68 in countries in Africa 70 in Uruguay and Argentina, 72 according to IN76 of MAPA (Brasil, 2018) and 78, 80°GL or higher by dairy industries in Brazil.

The statistical frequency distribution of the data of the minimum and maximum grades used in the alcohol test contained in the 87 articles (Table 3) showed median values of 70° and 83°GL; mean of 67.56° and 86.78° GL; 1° quartile of 68° and 80°GL; 3° quartile of 72° and 94,25°GL for minimum and maximum respectively. The most frequent values were 68°GL of minimum and 80°GL of maximum in the alcohol test.

Many studies used different alcoholic grades, from 64 to 80°GL, to characterize milk stability in surveys (Marques et al., 2011; Chen et al., 2014; Machado et al., 2017), while others evaluated milk stability using only one fixed grade for ethanol concentration (Marques et al., 2006; Zanela et al., 2009; Battaglini et al., 2013; Oliveira et al., 2020). The raise in ethanol concentration increases the likelihood of stability failure due to higher challenge to casein micelles (Fischer et al., 2012; Manske et al., 2020).

The value of 80°GL as the maximum value, along with the higher values, was less used. The rationale for using progressively higher grades refers to the ability of milk to better support more challenging industrial processes such as UHT, milk powder and evaporated milk. However, there is no defined linear relationship between resistance (absence of coagulation) at higher temperatures or for longer time with the ethanol concentration in the test (Molina et al, 2001), and there was no evidence of the need to test milk with alcoholic grades higher than 75 or 76ºGL in the case of UHT, as already suggested by Shew (1982).

Table 3. Frequency of minimum and maximum alcohol graduation values (°GL) used in 87 articles

Min	Frequency	%	Max	Frequency	%
0	1	1,6	74	1	2,7
2	1	1,6	75	3	8,3
20	1	1,6	80	11	30,5
30	1	1,6	82	3	8,3
40	2	3,2	84	3	8,3
50	1	1,6	85	1	2,7
60	1	1,6	90	1	2,7
62	1	1,6	91	1	2,7
64	1	1,6	92	1	2,7
66	5	8,1	94	2	5,5
68	13	21,3	95	1	2,7
69	1	1,6	98	2	5,5
70	9	14,7	100	5	13,8
72	9	14,7	110	1	2,7
75	1	1,6			
76	5	8,1			
78	1	1,6			
80	1	1,6			
95	1	1,6			
96	4	6,5			
100	1	1,6			

Discussion

World history on the evolution of the occurrence of milk with low stability

At the beginning of publication dates, there was a great interest in identifying raw milk with low stability to avoid problems during industrialization processes. In the study of Seekles and Smeets (1952), feed restriction and high levels of ionic calcium were related with low milk stability. The alcohol test gradually fell into disuse, because it could not identify the reason for low stability, along with the adoption of improved feeding practices, health and milk conservation in the developed countries that improved milk composition, animal health and stability (Horne, 2016).

In the last 20 to 30 years, countries that initially did not do as much research on the topic, stood out among the main countries that are dedicated to the subject of milk stability, such as Brazil, Ireland, Poland and China. In part, the continued use of alcohol testing in Brazil is due to the great variability in milk quality, the diversity of climate, cow breed, food and feed management practices, and conservation measures of raw milk, with predominance of family production systems (Gabbi et al., 2013; Werncke et al., 2016), in addition to the predominant use UHT as thermal raw milk processing. The publications evolved from surveys of stability values, assessing seasonal patterns (Marques et al., 2006; Zanela et al., 2009; Oliveira et al., 2011; Machado et al., 2017) to controlled experiments about factors affecting stability such as feeding practices (Marques et al., 2010; Fruscalso et al., 2013; Machado et al., 2014; Martins et al., 2019), genetics (Botaro et al., 2009; Fagnani et al., 2017), casein composition (Barbosa et al., 2012), lactation stage (Marques et al., 2010); heat stress (Abreu et al., 2020), health conditions (Marques et al., 2011; Fagnani et al., 2014). Also experiments including the mechanisms that could explain these results, such as permeability of tight junctions (Stumpf et al., 2013) and increase in titratable acidity due to heat stress (Abreu et al., 2021) were performed.

Ireland, as the second country in number of articles, addressed topics related to feed restriction (Gulati et al., 2019), supplementation (O'Brien et al., 1999), season effect (Lin et al., 2017), lactation stage (O'Connel, 2017), ionic calcium (Pyne & Mchenry, 1955). In Argentina, the study by Chavez et al. (2004) highlighted the main factors underlying heat coagulation time and alcohol tests. In Poland, studies related to milk agitation on physical and chemical characteristics stood out (Warminska et al., 2006; Czerniewicz et al., 2006). In Taiwan, Lin et al. (2009) evaluated seasonal variations in stability, showing that there is a greater chance of having milk with lower stability in summer and with higher levels of ionic calcium. The consistency of publications over the last century and the present, investigating why milk has its stability diminished shows us that this issue is a problem.

Relation of the results in the alcohol test and the alcoholic grades used

Historically, in the early 20th century, milk stability was tested using alcoholic solutions with 68°GL (Rathnayake et al., 2016), then, during the last decades of the 20th century, it was tested with 70 and 72°GL (Barros et al., 2001; Molina et al., 2001). However, since the beginning of the 21st century, industries have been increasing ethanol concentrations in the test solution to values progressively of 76 (Zanela et al., 2006; Marques et al., 2007), 78 (Machado et al., 2017), and even higher, as 78 (Machado et as., 2017) and 80°GL. The industry assumed that there is a positive linear relationship between milk stability and its thermal resistance, but this linear relationship was not been clearly proven (Molina et al., 2001; Machado et al., 2017).

It is observed that 50% of the publications considered in the present systematic review used alcohol levels between 68 and 72°GL, and that in 30.5% of the articles 80°GL of alcohol was used as the maximum degree of the test. Much is due to the difference between the alcohol grades used in the experiments, which are normally higher to test the maximum stability of the milk, but these grades are used by the industry to verify if the product will withstand heat processing only. However, it is known that increasing the concentration of ethanol in the test solution increases the frequency of positive results, that is, unstable milk (Fischer et al., 2012), as it will cause greater destabilization of proteins, due to the reduction of the dielectric constant of the medium and decreased repulsion of casein micelles, causing their coagulation (Horne and Parker, 1981). There is still a deficiency in determining the minimum alcohol concentration to test the suitability of milk for specific industrial processing. Shew (1982) indicated that stable milk in the 75°GL alcohol test could be used for UHT processing, which is an industrial product that subjects milk to high temperatures, requiring a high thermal stability of the milk to resist such a procedure without settling in the equipment. However, UHT equipment has changed since then and there are doubts about the minimum ethanol limit that should be used (Machado et al., 2017).

Manske et al. (2020), after comparing the alcoholic degrees between 72° GL and 80°GL, recommended the concentration of 76° GL, although not specific reason was provided. These authors reported higher percentage of positive samples in the alcohol test at the concentration of 78°GL, corroborating with Fischer et al. (2012), that as higher alcohol concentration was used in the test, then greater number of cases of unstable milk.

The choice between ethanol concentrations in alcohol testing can be influenced by the type of dairy product. Products such as UHT milk require greater stability, if its stability of raw is not adequate, the same coagulates and forms incrustations in the industrial equipment, which causes industrial income loss and higher costs to inspect and clean the equipment more frequently. However, studies relating the different ethanol concentrations of the test solutions and their influence on dairy products are still scarce in the literature, and it is not yet possible to present a position regarding this subject (Manske et al. 2020). Molina et al. (2001) correlated alcoholic grades from 68 to 78° GL with heat coagulation time test, and reported that the alcoholic grade of 75° GL would be recommended. The same authors did not find a significant linear association between alcohol concentration and heat coagulation time.

Factors affecting the stability of milk proteins

The main contribution of this study was the identification and organization of the 35 factors that affect the thermal stability of raw bovine milk mentioned in the literature and selected by this systematic review. Initially, the discussions will be made to clarify the links and relationships discovered by the analysis of the 87 articles identified by the systematic review. Historically, the main cause of the low thermal stability of milk was the excessive acidity acquired due to high bacterial load and/or due to inadequate cooling. Excessive acidity increases the concentration of ionic calcium, which in turn reduces the negative charges of casein micelles, decreasing the repulsion between them and favoring their precipitation (O'Connel & Fox, 1999; Horne, 2016).

In the second half of the 20th century, milk with low stability was observed, but with acidity within the normal range in countries such as the Netherlands, called Utretch syndrome (Sekles & Smeets, 1955). Changes of this nature were reported in Japan (Yoshida, 1980 cited by Ponce, 2000), Italy (Pecorari et al., 1984 cited by Ponce, 2000), Iran (Sobhani et al., 1998), Cuba (Ponce, 2000), Uruguay, (Barros et al., 1999), Argentina (Negri et al., 2001) and Brazil (Conceição et al., 2001; Donatele et al., 2001; Marques et al., 2007; Oliveira et al., 2007; Zanela et al., 2009; Oliveira et al., 2011, Machado et al., 2017).

According to Zanela et al. (2006), Unstable Non-acid Milk (LINA) is characterized by coagulating in the alcohol test without titratable acidity above 18ºD. The low stability of raw milk is affected by factors related to milk, animals and the environment, and also the type of test used (alcohol or heat) assuming a multifactorial character. Stability is related to the resistance of casein to precipitate, and basically depends on the concentration and balance between the various mineral salts of milk (Horne, 2016), that is, stability is related to milk proteins when undergoing heat treatments in industry.

The stability behavior of the milk expressed as ºGL of ethanol which causes visible clots as a function of pH variation, makes it possible to distinguish two types of milk. In the case of type A milk, there is a curvilinear response of increased coagulation time with increased pH, but in the usual pH range of the milk, there is a depression of the coagulation time. On the other side, type B milk shows an increase in the coagulation time with the increase of the pH, in a curvilinear way and without presenting a minimum stability value in the usual pH range of raw milk (6.6 to 6.8) (White and Davies 1958; Horne and Parker 1981). These differences in the type of milk (A and B) were attributed to different proportions between k-casein and α-lactoglobulin (Rose, 1961). Sweetsur and White (1974) confirmed that the type of milk has different coagulation times when subjected to the heating curve with the same pH.

The composition of milk, protein fraction and mineral salts affect milk stability, being related to breed, diet, season and lactation stage. The same animal, along lactation, presents changes in the milk inorganic constituents (Mitamura, 1937; O'Connel, 2017). The pH of the milk influences the chemical form of the minerals dispersed in the milk and their equilibrium. Donnelly and Horne (1986) observed that pH induces changes in the concentration of divalent cations, such as Ca^{2+} and Mg^{2+}. The pH reduction increases the concentration of ionizable and soluble calcium, reducing the stability of the milk in both tests, attributed to the lower negative charge between the casein micelles. The concentration and dispersion of mineral salts vary according to lactation stages and health conditions.

The relationship between milk salts or minerals such as calcium, magnesium, potassium, sodium, chloride, citrate, and phosphate with milk stability has been researched since the early 20th century. Since the first publication considered in this review (Sommer and Binner, 1923), calcium has been considered as the main mineral that affects the stability of milk to alcohol testing. Seekles and Smeets (1952) attributed the low stability of milk to the alcohol test to the excess of calcium ions, e.g., reported in Utrecht's Syndrome. According to these authors, the increase in calcium levels could be a consequence of the increase in the level of soluble calcium, the reduction of the citrate and of soluble phosphate, since both citrates and phosphates bind to calcium, decreasing the concentration of ionic calcium. Pyne and Mchenry (1955) suggested that the concentration of calcium ions and colloidal phosphate of the caseinate-phosphate complex of milk are the two main factors that determine the tendency of milk to coagulate in the heat coagulation time test. With increasing concentration of ionic calcium, it moves from the soluble phase to the colloidal phase, reducing the stability of casein (Silva, 2004). Thus, if the concentration of calcium ions and colloidal phosphate is relatively high, with only a moderate degree of acidity development the denaturation of casein occurs, making coagulation quite fast.

Horne and Parker (1981) also suggested that soluble calcium is the dominant factor in milk stability. The formation of calcium phosphate, as the pH increases, increases the stability of the milk and thus, higher alcohol concentrations are necessary to induce coagulation. This fact explains why milk tends to have greater stability when testing with alcohol at higher pH. Tsioulpas et al. (2007) showed a nonlinear negative relationship between the concentration of free calcium ions and the stability of milk in the alcohol test (r = -0.84). Lin et al. (2009) showed that milk samples with low stability to the alcohol test had significantly higher total and ionic calcium concentrations than medium and high stability samples. Singh and Fox (1987) found that changes in soluble calcium and phosphate levels markedly affected the dissociation of k-casein, where the reduction of phosphate concentration or the increase of calcium concentration reduced the stability of the milk when testing with heat coagulation time test. In addition to phosphates, citrate also acts by sequestering ionic calcium, improving milk stability (Tsioulpas et al., 2007).

Not only does calcium have an effect on milk stability, but also the natural variations between citrate and calcium, magnesium, phosphate and sodium in milk also interfere with stability, and are closely linked to the structure of casein micelles. Akkerman et al. (2019) found a positive correlation between complex serum calcium and citrate, and the higher the citrate content, the greater the stability of the milk, according to the results of Gaucheron (2005). According to Sommer and Hart (1919), in unstable milk samples there was excess calcium and magnesium in relation to citrates and phosphates. Citrate is known to be a stabilizing agent by reducing the concentration and ionic calcium of milk (Santos & Fonseca, 2007). According to Rose (1962), colloidal phosphate decreases milk stability in the thermal coagulation time test. According to Singh & Fox (1987), calcium and sodium ions, because they are positive, interact with negatively charged phosphates present in casein micelles and destabilize them. On the other hand, the levels of sodium and potassium have a positive correlation with the osmotic balance of the blood, showing that if these levels are altered, there is a greater passage of ions to the milk, which can lower the stability of the casein of the milk (Tsioulpas et al., 2007).

In addition, the characteristics of micelles and submicelles can affect milk stability. For example, casein micelles with smaller size and diameter present greater stability to heat treatments (O'Connell and Fox 2000), probably related to increased concentration of κ-casein and reduction of α-casein/β-casein ratio (Davies and Law, 1980; Mcgann et al., 1980). In addition, the smaller micelle size may be related to the K-casein glycosylation process. According to Martins et al. (2019), animals fed diets with low Rumen Degradable Protein Ratio compared to Non-rumen Degradable Protein (PDR:PNDR) showed a higher glycosylation rate of k-casein, (binding of sugars in their structure, mainly galactose, N-acetyl galactosemia or neuraminic N-acetyl acid) (Fox & Mcswenney, 1998). This increase in k-casein glycosylation was associated with the formation of smaller micelles (Bijla et al., 2014), increasing the coagulation time in the thermal coagulation time test (Glantz et al., 2010). There is a correlation between maximum and minimum thermal stability and the β-lactoglobulin content (r = +0,50 and -0,52 respectively) (Rose, 1962), i.e., the higher the β-lactoglobulin content, the greater the milk stability for the thermal coagulation time test, however, the author did not clarify the mechanism as such an event takes place.

Regarding the chemical composition of stable and unstable milk in the alcohol test, there is no consensus among the various studies on unstable and stable milk composition. Some studies reported an increase in fat concentration (Oliveira and Tim, 2006; Marques et al., 2007; Zanela et al., 2009), while other studies found no differences in fat concentration (Barbosa et al., 2009). Protein concentration reduction was detected (Ponce and Hernández, 2005; Zanela et al., 2006; Marques et al., 2007), but other studies did not find differences in relation to protein concentration (Oliveira and Timm, 2006; Fagnani et al., 2016). A little more consistently, lower lactose values were reported in unstable milk samples (Marques et al., 2007; Fruscalso et al., 2013; Stumpf et al., 2013), while others found no differences (Lin et al., 2009). Part of these studies evaluated milk stability during diet or nutritional restriction, in part explaining the results of unstable milk with lower lactose levels. This lack of consensus on the milk composition has multiple explanations, among the most common being the nutritional status of cows, the type of feeding and the lactation stage. However, the lack of clear relationships or the existence of complex relationships between milk components and milk stability, a fact already reviewed by Horne (2015).

Milk composition and stability vary during the lactation period of dairy cows. Gulati et al. (2019) found no statistical difference in the heat coagulation test in milk samples collected at the beginning, middle and end of lactation, but observed a tendency for milk samples collected at the start of lactation to coagulate in less time during heating. In a study evaluating the composition of milk between 1 and 90 days after calving, Tsioulpas et al. (2007) observed that stability in alcohol test progressively increased, reaching values above 72% between 7 and 15 days after delivery, while the concentration of ionizable calcium has been reduced to less than 2 mmol from 15 days after delivery, coinciding with titratable acidity values below 18°D. According to Mitamura (1937), 52% of the samples of raw milk from cows at the end of lactation tested positive for alcohol 70°GL, as did Donnelly & Horne, 1986; Horne et al., 1986; Marques et al. (2010) and O'Connell et al. (2017).

White and Davies (1958) observed lower values of milk stability at the beginning and end of lactation, which coincided with higher concentrations of ionized calcium and soluble magnesium, and possibly the lower pH values of lactating milk of 6.5 to 6.7. However, milk samples at the end of lactation presented higher pH, between 6.8 and 7.0, and possibly another mechanism may be influencing stability reduction. The higher salt concentration in cows' milk at the end of lactation is probably due to increased permeability tight junctions of mammary epithelial cells, increasing the influx of sodium and chlorides, but this mechanism has not been proven. However, this relationship between lactational stage and stability was not confirmed by other authors, such as Barbosa et al. (2009) and Barlowska et al. (2014). On the other hand, the mechanism by which milk has its reduced stability at the end of lactation has not been determined, not even the moment when stability is reduced.

The level of milk production of the animal may also be indirectly associated with milk stability. According to Janu et al. (2007), animals with high production compared to those with average production produced milk with greater stability in the alcohol test, respectively, 71.9 and 63.9° GL, corroborating the results reported by Manske et al. (2020). One of the reasons given is the quality of the diet of the animals, so that they can present high milk productivity, that is, it does not necessarily demonstrate that the productive potential can affect stability directly, but rather a direct relationship with the diet. On the other hand, Doran et al. (2020) failed to demonstrate the effect of genetic merit for milk production on milk stability.

The physiological and pathological conditions of cows have been mentioned for inducing changes in the chemical composition of milk. According to Hernandéz and Ponce (2005), the occurrence of Abnormal Milk Syndrome (AASI), a milk with low stability and normal pH and acidity, was related to anemia and metabolic acidosis in animals. Fagnani et al. (2014) observed that animals with some type of disorder in their acid-base balance presented a higher occurrence of unstable non-acid milk. Marques et al. (2011) induced metabolic acidosis by supplying ammonium chloride to lactating cows, and verified lower milk stability at alcohol testing and higher concentrations of ionic calcium in the milk of cows supplemented with anionic salt (DAD -200meq/kg MS of the diet) in relation to the control cows (DAD +100 meq/kg MS of the diet). According to Cobellini (1998), the increase of fixed anions (Cl-, SO4-2) in the diet by the administration of so-called "anionic salts" increases the systemic concentration of the hydrogen ion (H+) evaluated in the animals' urine. To keep homeostasis and blood pH within normal values, the animals increase the bone reabsorption of calcium, which may be related to the increased of ionic calcium in milk (Marques et al., 2011). Later, Martins et al. (2015) when evaluating anionic salt doses in the diet of lactating cows observed that the ionic calcium and κ-casein concentrations in milk were reduced as positive dietary loads increased (from negative to positive), increasing the stability of the alcohol-testing milk and the stability of the milk during heating to 140 ºC. Regarding mastitis, there was no change in milk stability and there was also no negative relationship between mastitis or somatic cell count (SCC) and milk stability (Fischer et al., 2012). The results of Martins et al. (2015) and Machado et al. (2017) corroborate those of Fischer et al. (2012). However, Chavez et al. (2004) found lower SCC values in unstable samples in the alcohol test, and Oliveira et al. (2011) found the opposite.

It is known that the difference between the breeds of the animals causes differences in the composition of the milk, even if those animals are under the same system and feeding effect, but can also have an effect on the stability of the milk. Mitamura (1937) analyzed samples of fresh milk from Holstein, Ayrshire and Guernsey cows, and reported that Holstein cows showed higher coagulation frequency when tested for alcohol, and coagulated with lower alcohol content (78 °GL) compared to cows of the Guernsey and Ayrshire breeds, which coagulated with 79 and 81°GL in the alcohol test. According to Botaro et al. (2009), the milk of Holstein cows presents greater stability to the alcohol test than the Holstein x Gyr (Girolando) cows, although the authors have not made clear the mechanism of such difference. Barlowska et al. (2014) found that the milk of the Simmental cows, compared to that of the Polish Holstein and Jersey cows under the same conditions, had greater stability in the heat coagulation test.

Genetic polymorphism, related to the genetic frequency of the animals, was detected for all major milk proteins (Eigel et al., 1984) and affected milk production and composition (Mclean et al., 1984; Ng-Kwai-Hang et al., 1984, 1986; Lin et al., 1989) and milk stability (Mclean, 1987; Mclean et al., 1987). Cows with different genotypes of k-casein AA, AB or BB have different milk stability. According to Imafidon and No-Kwachang (1991), milk containing the BB phenotype for k-casein has higher calcium levels and a lower concentration of beta-lactoglobulin (β-LG), presenting greater stability in the test of thermal coagulation time compared to milk with AA phenotype, as well as Paterson et al. (1999). Conversely, Robitaille (1995), when comparing the mean values of stability of the milk of each individual with k-genotypes casein AA, AB and BB suggested that the best genotype for k-casein in terms of thermal stability was AA. Moreover, Botaro et al. (2009) did not find an association between thermal stability of milk and k-casein genotypes.

The saline constituents of the blood and their relation to the thermal stability of the milk are still little studied. According to Mitamura (1937), the saline constituents of the blood have no relation to the variation of the results of the alcohol test. Conversely, Sobhani et al. (1998) compared blood samples from cows producing stable and unstable milk to the alcohol test and reported differences ($P < 0.05$) in potassium, chlorine, glucose concentrations and pH in blood. But there was no difference ($p > 0.05$) in the concentrations of calcium, magnesium, phosphorus, sodium and urea in blood. According to these authors, low blood glucose levels (39.8 mg / 100ml) may probably be the original factor for the incidence of milk instability, but such a statement requires further investigation.

Feeding was pointed out as one of the main factors affecting milk stability in the literature, in addition to being related to other factors such as the season affecting the quantity and quality of food available to lactating animals. Feed restriction is a factor related to the absolute or relative lack of nutrients that outweigh the needs of animals, influenced by animal management, farm infrastructure, as well the effect of the season (certain moments of food shortages) and thermal stress (causing the animal to seek or eat less food), that can reduce milk stability. The feed restriction was mentioned as a probable factor responsible for the reduction of milk stability by Seekles and Smeets (1952), when researching the so-called Utrecht anomaly". In addition, the authors related it to excess calcium ions, as low stability happened either in situations of excess of food, between the years 1927 to 1939, or in situations of shortage of food, reported during the Second World War period on French territory. The higher occurrence of LINA in periods with food shortages was also reported by other authors. Bataglini et al. (2013) reported higher occurrence of LINA in the fall (62.8%) and lower in the winter (26.7%), in agreement with the results of Marques et al. (2007) and Zanela et al. (2009). The lower availability of nutrients, or excess of fiber, was related to the lower stability of milk (Barchiesi et al., 2007). In addition to these surveys, other studies directly evaluated experimentally the effect of reducing food supply (Okada et al., 2001; Zanela et al., 2006; Marques et al., 2010; Barbosa et al., 2012; Stumpf et al., 2013; Fruscalso et al., 2013) and the supply of nutrients. These aforementioned studies showed that the reduction in food and nutrient intake depressed the thermal stability of milk. However, there are contradictory results, such as those presented by Barbosa et al. (2012), that subjected cows to 40% feed restriction and reported no significant decrease in milk stability compared with cows not feed-restricted. This contradictory result might be explained by differences in feeding practices used in those trials, such as severity and duration of feed restriction as well stage of lactation, between others (Gabbi et al., 2015).

The effect of the season on milk stability may also be related to the quantity and quality of food, especially in situations where animals are fed on pastures. Some authors observed lower values of milk stability in autumn/winter/spring (Donnelly and Horne, 1986; Oliveira and Timm, 2006; Marques et al., 2007; Zanela et al., 2009; Barlowska et al., 2014; Manske et al., 2020), which may be associated with the transition to summer grazing, with less availability of forage and poorer in some nutrients. Barchiesi et al. (2007) observed a lower stability to the alcohol test during the summer, a season with lower precipitation, and forage usually has higher fiber content. The stability of the milk in the alcohol test followed the same seasonal trend of the thermal coagulation time, with values significantly higher in spring than in autumn, caused by the improvement of forages (Chen et al., 2014). However, Oliveira et al. (2020), when evaluating the occurrence of LINA in the state of Minas Gerais, Brazil, found that more samples were positive for the alcohol test (n=10,561) at the end of winter and spring/summer in the region due to low forage availability and increased feed restriction. On the other hand, Lin et al. (2017) did not observe differences in milk stability between seasons.

The amount and type of nutrients available in the diet also affects milk stability. The stability of alcohol-tested milk increased from 74.2 to 76.4 °GL, when the concentration of total digestible nutrients (TND) reached 79% of the estimated needs (Gabbi et al., 2018). It is necessary to recognize the great plasticity of the responses of cows in relation to the composition of the diet, such as the results reported by Machado et al. (2014), who offered different proportions of concentration in the diet: 33, 45 and 55%, with no added rumen buffers or alkalizing compounds, and no differences in milk stability were observed. For Voges et al. (2018), the use of forages and concentrate supplements with consequent improvement of animal productivity and higher lactose content in milk were associated with lower occurrence of milk with low stability to alcohol testing in southern Brazil.

According to Marques et al. (2010), supplementation with adequate energy and protein levels in the diet of the animals was more efficient in improving milk stability to alcohol test, compared with low levels of supplementation or only high levels of protein. O'Brien et al. (1999) compared cows in pasture with restricted pasture availability, standard pasture availability and standard pasture availability + supplemented with 3 kg of concentrate, reporting an increase in stability to alcohol test as the availability of pasture and supplementation increased. One possible explanation for this may be the influence of the excess of degradable protein on the diet, reducing κ-casein glycosylation evidenced by Martins et al. (2019), in diets with excess of rumen degradable protein.

The composition of the nitrogen fraction of feeds can alter the flow of ammoniacal and non-ammoniacal nitrogen to the intestine, and thus, alter the proportion of amino acids available for the synthesis of milk proteins. According to Barbosa et al., (2012), milk samples unstable to the alcohol test had lower concentration of κ-casein, but higher concentration of β-casein than stable samples. Martins et al. (2021) evaluated the inclusion of non-fibrous carbohydrate sources in the diet and the inclusion of buffering on milk stability in dairy cows, by partially replacing the ground maize by citrus pulp and including buffers in the diet. These authors reported lower heat coagulation time, along with lower casein values. However, there was no effect on the ionic calcium concentration and stability to the alcohol test.

Zinc in the diet of lactating animals has been cited as a possible factor affecting milk stability. Usually, zinc is known to play an important role in immunity, reproduction and hormonal activities in dairy cattle (CRN 2001). At the cellular level, it is essential to maintain the epithelial barrier by regulating proteins from firm junctions of the mammary gland (Miyoshi et al., 2016). Singh et al. (1989) demonstrated that the nature of zinc binding to casein micelles makes them very stable, dissolving only at pH 6.6. It is known that the increased supply of zinc in the diet can increase the concentration of zinc in dairy cows' milk (Pechová et al., 2006). However, Shaffer et al. (2018), the only authors to evaluate the effect of zinc on milk stability in lactating cows, found no evidence that zinc supplementation, using an inorganic or complexed with methionine sources, affects mammary gland epithelial cells integrity and milk stability when testing with the time of thermal coagulation in healthy lactating cows.

The effect of high temperatures causes heat stress in animals, a fact that can negatively interfere with milk stability. Reports of reduced milk stability in both tests during the summer have been increasingly common (Abreu et al., 2020), probably related to thermal stress. Exposure to temperatures above the range of thermal comfort and solar radiation causes a number of changes in cows' metabolism, such as ruminal and metabolic acidosis, dehydration and increased plasma cortisol, reduce blood consumption and flow to the mammary gland and increase apoptosis of mammary epithelial cells (Tao et al., 2018) with a dramatic drop in milk production and a variable change in its components (Bernabucci et al., 2010; Cowley et al., 2015; Tao et al., 2018). Severe thermal stress increased the acidity of milk (Abreu et al., 2020), which may partially explain the lower stability of milk observed in cows without access to shade. The higher acidity observed may be related to the higher concentration of ionic calcium in milk, an important factor that regulates milk stability (Chávez et al., 2004; Horne, 2015). According to Lin et al. (2009), there was a higher incidence of milk with low stability in the alcohol test (coagulation of samples using less than 70°GL) in summer, coinciding with heat stress in animals.

The effect of temperature and storage time influences the thermal stability of milk. Long storage periods of raw milk even at low temperatures induce changes in almost all its components, regardless of milk microbiology (White, 2003; Harding, 1999; Stepaniak and Rukke, 2003). According to Czerniewicz et al. (2006), milk samples stored at temperatures around 4°C showed less significant physicochemical changes. Davies and White (1966) point out that the time of thermal coagulation increased dramatically when milk was kept at a temperature of 4 °C. After storing the raw milk obtained from 2 milkings at 4 °C for 6 days, Guinot-Thomas et al. (1995ab) reported declines in pH, milk nitrogen, β-casein and colloidal levels of calcium and phosphorus, while NPN and γ-casein levels increased. The reduction of thermal coagulation time related to proteolytic and lipolytic activity occurs due to an extensive dissociation of β-casein and colloidal calcium phosphate from micellar casein during refrigerated storage (Dalgleish and Law, 1989; Raynal and Remeuf, 2000). Another aspect concerning storage conditions is the effect of light exposure. Milk exposed to light storage increased the time of thermal coagulation (Sweetsur and White, 1975), an effect attributed to the catalytic action of light on riboflavin, increasing the k-casein/β-lactoglobulin ratio and thus, the thermal stability of milk.

Agitation during transport, even when thermal and sanitary conditions are adequate, is accompanied by mechanical interactions, mainly shocks and vibrations that occur due to the movement of fluids during transport, which have a negative effect on specific components and on the final quality of the raw material (Hyslop & Fox,1981; Palich, 1993; Warmiñska et al., 2003). According to Czerniewicz et al. (2006), lipolytic and proteolytic processes augment with the increase in frequency and vibration time. Warmiñska et al. (2006) submitted milk samples to a vibration with acceleration of 1g and vibration frequency of 10 to 60 Hz (Warmiñska and Kruk, 2001). These authors reported that vibrations of 120 minutes at a frequency of 60 Hz resulted in a reduction of milk coagulation time in 40%, and in more coagulated milk samples with lower alcohol values in the alcohol test, negatively affecting the technological usability of milk. Angelo et al. (2018) compared milk samples from a manually stirred storage tank (after the refrigerator shovel remained for five minutes, the milk was stirred vertically using a metal stirrer) and automatic stirring (The stirrer was activated every hour, remaining active for 15 minutes), and reported that the samples in automatic stirring had lower values of stability in the alcohol and acidity test compared to manual stirring.

In general, the variables of the farm infrastructure, such as the total area, area for the feed production for the animals, in addition to the appropriate use of milking practices and level of education of the owners, are related to the stability of the milk (Gabbi et al., 2013, Werncke et al., 2016). According to Werncke et al. (2016), in the group of producers with the highest technological level, the stability of milk to alcohol test was higher, due to the best practices of nutrition, hygiene of animals and facilities. Similarly, Manske et al. (2020) found that the smaller the total area destined for food, the greater the probability of occurrence of LINA, related to the ability to provide food.

Thermal processes and stability

The use of thermal treatments, necessary for the transformation of the raw material into industrial by-products, can affect the stability of the milk. Tissier, Lalande and Corrieu (1984) pointed out that during the heating process, using temperatures of 90°C, β-lactoglobulin denaturation occurs, and that at temperatures above 130°C, there is denaturing of β and α- casein and, consequently, these proteins can be deposited in heating equipment. According to Grandison (1988), during UHT heating processes, destabilization of milk solids, mainly fatty cells and casein micelles, may occur, generating sediments on the equipment surface, increasing the need for cleaning and reducing the efficiency of these industrial processes. The mineral composition of milk, mainly calcium and citrate, affects the resistance of milk to the heating process (Karlsson et al., 2019). Even with small increases in ionic calcium and ionic magnesium concentration, the running time of the UHT plant is reduced (Grandison, 1988). This decrease in thermal stability is caused by the diminished repulsion between negatively charged casein micelles, leading to an increase in intermicellar interactions (Crowley et al., 2014).

Other types of heat treatments, which increase the shelf life of dairy products can affect milk stability, such as high-pressure (HP) and high-intensity thermal and non-thermal ultrasound (HIUS). In dairy foods it is usual to apply 100-400 Mpa for 5 to 10 minutes (Myllymaki, 1996). In one of the first studies of the use of HP treatment in dairy products, Schmidt and Buchheim (1970) observed a decrease in the size of casein micelles after milk treatment with electron microscopy. Similarly, the application of pressures between 250 and 600 Mpa reduced the size of the casein micelle (Desobry-Banon et al., 1994; Gaucheron et al., 1997; Needs et al., 2000). On the other hand, Huppertz et al. (2003), when using treatment applying 250 Mpa, observed an increase in the micelle size, but under pressures equal to or greater than 300 Mpa, micelles have their size reduced to more than 50% of the original or are dissociated. The use of HP with high pressures such as 600 Mpa in milk with pH superior to 6.7 increased the stability of the milk to the alcohol test.

The treatment of milk using high-intensity non thermal and thermal ultrasound (HIUS) becomes an alternative that does not affect the physico-chemical and microbiological changes in liquid products such as milk due to the phenomenon of acoustic cavitation. Scudino et al. (2020) reported that the stability of raw milk using HIUS technology showed similar to conventional heat treatments, such as UHT, preserving the physical, chemical, nutritional and sensory characteristics of milk, without affecting its stability.

Tests to assess the stability of the most commonly used milk proteins

There are several tests used to estimate the thermal stability of milk. These include alcohol tests (also referred to as ethanol testing, or alizarol if it contains alizarin, a pH indicator). The test dehydrates the casein micelles and reduces negative charges, and thus, the repulsion between casein micelles. Other tests apply heat to milk samples, such as the boiling test, that consists of heating milk samples at a temperature up to 100 °C, and considering positive results when clots are visually detected (Lanara, 1981). Moreover, the heat coagulation time test warms the milk placed in glass capillaries at 140ºC (in an oil bath), and records the time necessary for coagulation to occur. Alternatively, there is the coagulation temperature test, which records the temperature at which visible clots formed.

Milk samples that have taken longer to coagulate or coagulate at higher temperatures are considered more stable. There is no clear-cut point or minimum time that milk should last to be considered stable, besides requiring special facilities and equipment (Machado et al., 2017). According to Singh (2004 that there is no ideal method to accurately assess the resistance of milk to heating in the equipment and conditions used by the industries, except the use of experimental plants. Historically tests have been used to ensure the quality of raw bovine milk since the 19th century, as evidenced by Sommer and Binner (1923), who cited the use of the alcohol test in 1890, at that time being used to detect excessive acidity, unwanted clotting and other undesirable characteristics. There is currently no availability of fast, inexpensive and practical tests to assess milk stability in the face of industrial warming (Machado et al., 2017).

Other researchers have evaluated other tests, such as the one described by Ramsdell et al. (1931), testing the precipitating action of some salts to evaluate the resistance of milk under thermal sterilization processes and transformations into evaporated and condensed products. These authors found that monobasic potassium phosphate was considered the most satisfactory precipitating agent. The coagulation was caused by the combined action of added salt and heat, and when tested in an industry in California, the milk that coagulated in this test also showed low resistance to heating.

Pyne and Mchenry (1955), analyzing the time of coagulation of milk by heat, found that acidity increased during the heating process, mainly following the thermal decomposition of lactose, generating acid compounds such as acetic acid, levulic, formic, pyruvic, hydroxymethyl furfural, aldehydes, alcohols and reductants. In addition, phosphate released from denatured casein and, to some extent, phosphate balance displacement, and partial denaturing of caseinate may contribute to acidification by a change accompanied by increased protein sensitivity to calcium ions, which could compromise the method. Recently, Hanuš et al. (2021) reported that the stability of milk to test the coagulation time by heat was influenced by farm characteristics and meteorological conditions.

Chavez et al. (2004) evaluated the stability of raw milk sampled in dairy silos and compared heat coagulation time and alcohol tests. These authors found that most of the factors affecting these tests are not the same, except for pH (pH induces changes in milk mineral profiles, mainly divalent calcium, Donnelly and Horne, 1986) and ionic calcium (this affects the stability of the links between the casein, Silva, 2004). Carrying out the coagulation test requires equipment and facilities, time training of the employees, and does not provide a clear-cut value for the acceptance or rejection of milk. In this sense, according to Machado et al. (2017), there is still no other test (fast, clear-cut value, low cost, without demands of facilities and sophisticated equipment) to replace the alcohol test.

These tests (boiling, alcohol and heat coagulation time) are partially affected by different factors and also promote different challenges to casein micelles, resulting in null or weak correlations between them, as evidenced by Molina et al. (2001), Chavez et al. (2004) and Machado et al. (2017). This lack of relationship between the tests is explained by the different challenges to casein micelles by each one. The heat coagulation tests the protein denaturation point at a given temperature and the alcohol test promotes dehydration, affecting negative charge. Many authors use two or more tests, most commonly using alcohol testing and heat coagulation testing. It is recognized that these tests are not specific and do not discriminate against the cause of low stability.

The boiling test presents very low sensitivity, especially if the milk does not have excessive acidity. O'Connor (1994) and Kassa et al. (2013) indicated that the alcohol test is more sensitive in detecting the acidity of milk than the boiling test, because in the heating tests there may be an induction of occurrence of chemical reactions, some advantageous, in relation to urea of milk, but other deleterious ones, such as those that occur in relation to whey proteins and lactose. In other words, according to Horne (2016), milk that would likely present heat stability problems would also fail the ethanol stability test, that is, the use of one or more tests would only be a proof to give greater reliability to the results, because the alcohol test is a good indicator of thermal stability. These reactions proceed at temperature-dependent rates, which, if performed in a narrower range, allow a similar behavior to ethanol stability (Horne & Muir, 1990).

New technologies related to stability

Some studies have brought novelties on the inclusion of substances that can affect milk stability, such as the enzyme transglutaminase (Tgase). The treatment of milk with the enzyme transglutaminase (Tgase) creates intermicellar covalent cross-links (Sharma et al., 2001; O'Sullivan et al., 2002) by means of a transfer through the reaction between the carboxyl group of a peptide bound to glutamine residues and a-amino group of lysine (Zhu et al., 1995). Milk stability is increased as a result of Tgase treatment (O'Sullivan et al., 2001; O'Sullivan et al., 2002) probably due to polyelectrolyte brush crosslinking on the micellar surface, avoiding heat-induced dissociation of k-casein (O'Sullivan et al., 2002; Huppertz et al., 2007). According to Tarapatskyy et al. (2019), Tgase contributes to the formation of high molecular weight polymers in the 55-200 kDa range, and these modifications in the cross-bonds of milk proteins significantly increase the stability of milk to alcohol testing.

Kassa et al. (2013) studied the use of the enzyme lactoperoxidase (LP), but after detailed analysis of the full text and data contained in the studies, it was found that the use of LP extended the shelf life of fresh milk kept without refrigeration for more hours, but that the results did not relate the effect of the LP on the thermal stability of the milk, but rather to the low stability related to the acidity caused by insufficient cooling of the samples. Fagnani et al. (2016) measured the crystallographic profile of lactose from unstable and stable milk samples at alcohol testing, and showed differences between unstable and stable milk at alcohol testing, but to our knowledge it still is not recommended for a test for the dairy sector.

Conclusion

The low stability of raw bovine milk and the occurrence of phenomena also called Unstable Non-acid Milk (LINA), Abnormal Milk Syndrome (SILA) and Utrecht Anomaly, that even each of them presenting particularities and being different from each other, both affect milk stability, and also have multifactorial cause. It is known that milks with lower stability present higher prevalence in properties with lower infrastructure, varying according to the season, worsening during periods of food shortage, nutrient imbalance and thermal stress. The composition of the unstable non-acid milk is frequently similar to normal milk, and there may be a lower concentration of lactose, even with low somatic cell counts. Cow's health conditions such as metabolic and ruminal acidosis reduce stability, but high somatic cell count does not clearly influence milk stability. Inadequate hygienic conditions, by increasing bacterial contamination, reduce milk stability by reducing its pH. No other more specific, rapid and low-cost tests have been found to assess milk stability and doubts remain about the appropriate minimum ethanol concentration in the solution, in order to avoid the rejection of milk of acceptable quality which could be used by the industry. There is no evidence that values higher than 72 °GL should be used for milk screening on the farm, with the exception of milk destined for UHT, which should probably be stable in the concentration of 76 ºGL.

Supplementary Material

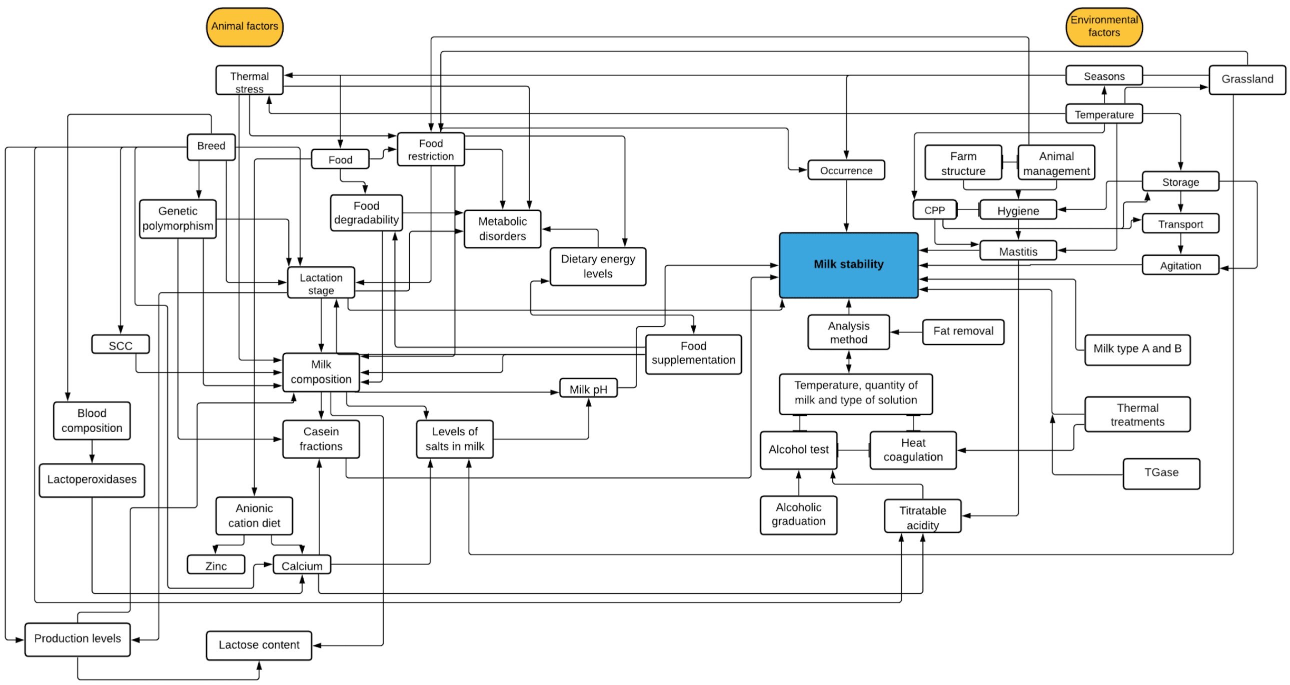

Figure 4 . Mind map on factors that affect milk stability

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CAPÍTULO III

Seasonal stability of raw bovine milk - a quantitative analysis on a systematic review

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Abstract

Seasonal variations in milk composition have been reported by many authors, and such differences have multifactorial causes (availability and quality of food, temperature conditions, photoperiod, lactation stage and birth concentration). The purpose of this study was to identify, critically evaluate and summarize the available information from the literature on the effect of the seasons on the thermal stability of raw bovine milk and milk composition through the results obtained in a systematic review of the literature. Based on the results of the systematic review, 7 articles met the selection criteria to perform a reanalysis of their data. The criteria for inclusion of the articles were: evaluation of milk stability and data obtained in different seasons of the year and presented in a chronological or monthly form that can be transformed into chronological seasons. During the spring, the milk was more stable, coagulating in test solutions with higher ethanol concentration (84.73°GL compared to the autumn, when the milk was less stable (81.18°GL). Winter and summer did not differ statistically from the others. Only protein, fat and lactose concentrations were positively correlated with stability. As the stability of the milk increases by 1 unit the alcohol level of the alcohol test, the fat, protein and lactose concentrations increased by 9.9, 14.5 and 18.1%, respectively. The concentration of casein tends to increase with the increase of milk stability. There is seasonal variation in milk stability, and the highest values coincide with the months of higher nutritional intake and lower thermal stress.

Key Words: alcohol test, meta-analysis, milk stability, raw milk, season.

Introduction

Seasonal variations in milk composition have been reported by many authors (Grandison et al., 1984; Horne, 1986; Auldist et al., 1998; Lock and Garnsworthy 2003; Gaucher et al., 2008; Lin et al., 2017), attributed to the availability of food and nutrients (Hernández & Ponce, 2005; Barchiesi et al., 2007), climatic conditions and the direct effect of thermal stress, which modifies the consumption and physiological and metabolic aspects of animals (Abreu et al., 2020). These changes in nutrient intake and animal metabolism may change the characteristics of milk processing, such as its stability, which can be evaluated by the ethanol resistance test in several countries such as Brazil (MAPA, 2019, IN76 and 77), Argentina, Uruguay, Russia, Cuba, Taiwan, India and South Africa (Machado et al., 2017; Martins et al., 2015). The stability of milk to ethanol (Milk Ethanol Stability - MES) was defined as the minimum concentration of added aqueous ethanol capable of inducing milk coagulation (Horne & Parker, 1980).

Several studies that monitor the variations in the stability of raw milk found a reduction in stability when animals face feed restriction situations due to the reduction in the availability of pasture (O'Brien et al., 1999; Fruscalso et al., 2013), nutrient supply (Marques et al., 2010; Gabbi et al., 2015), increase in the fiber content of pastures and forages used in food (Barchiesi et al., 2007), and thermal heat stress (Abreu et al., 2020). According to Marques et al. (2007), Zanela et al. (2009) and Bataglini et al. (2013), the occurrence of milk with low stability to the alcohol test was higher in the fall and lower in the winter, probably due to the lack of forages that occurs between the end of summer and winter.

Due to the heterogeneity in the results, it was sought to organize, through a systematic review of the literature, the reasons why the seasons and the changes they cause in the animal, can negatively impact the stability of raw milk bovine. The systematic review is a critical analysis of the current stage of scientific production of its theme, to identify gaps, consensus and controversy on the subject and insert the research problem in a path not yet covered by other authors (Brizola & Fantin, 2016). After a review, the authors usually resort to a statistical summarization and reanalysis of the data, a procedure that combines the results of several studies to make a reproducible and quantifiable synthesis of the data (Lovatto et al., 2007).

The purpose of this study was to identify, critically assess and summarize the available information in the literature on the effect of the seasons on the thermal stability of raw bovine milk and correlate the effect of stability with milk solids through the results obtained in a systematic review of literature.

Material and methodology

This article is the result of a systematic review that gave rise to another article, so the initial methodology of searching and filtering articles (systematic review of the literature) is the same for both, thus being the basis for the elaboration of both.

Research question

This study identified the effect of the seasons; summer, autumn, winter, spring, dry season and rainy season (corresponding seasons according to location on the globe), on the thermal stability of bovine milk to alcohol testing. This analysis is the result of a systematic review, whose objective was to evaluate the main factors identified as a cause of decreased stability of raw bovine milk. After the statistical analysis, it was found that the most mentioned factor was the effect of the season on milk stability.

Research methods to identify studies

The search strategy in the literature was defined based on the main concepts in terms of PIC'o: population (P), intervention (I) and context (C). Then, the PIC'o of the following study resulted in: POPULATION (population) - milk, being samples of individual animals and/or whole milk, on the farm; INTERVENTION (intervention/interest) - related to milk stability, to LINA, to alcohol or alizarol testing, heat; CONTEXT (context) - the factors that have already been mentioned that can negatively affect milk stability. Subsequently, the search key was elaborated, which consisted of the following sequence: ("milk") AND ("LINA" OR "unstable" OR "Stability" OR "Ethanol" OR "Alcohol" OR "non Acid" OR "Heat") AND ("feed Restriction" OR "Thermal stress" OR "Heat stress" OR "Lactation Stage" OR "Metabolic disease" OR "Metabolic disturb" OR "Digestive disease" OR acidosis OR mastitis OR agitation OR Storage OR transport).

In the Scopus and Pubmed database the advanced mode was searched and in the Web of Science database the basic search was used. The initial selection without duplicates had 2,774 records. After their eligibility analysis and selection by 3 observers, 30 selected records were obtained. However, after a request from an expert on the subject, 23 more records were included, totaling 53. In the review of the references of the 53 records, a further 31 records were added, totaling 84. On October 27, 2020, after the end of the selection of articles and their bibliographic references, a second search of bibliographic records was performed, which resulted in the insertion of three more records, totaling 87 records considered for statistical analysis.

Criteria for the selection of studies

With the a priori withdrawal of the duplicate articles, the eligibility of the articles was initiated, proceeding the evaluation of the summaries independently by three observers. Abstracts were selected for subsequent inspection if they met the criteria:

A) scientific articles and/or short papers published in journals;

B) have access to the full text;

C) have worked with raw bovine milk, individual or tank milk and may be defatted for analysis;

D) contain the word "stability" related to raw milk;

E) have worked with alcohol testing and alcohol graduations or thermal stability by heating and clotting time;

F) have used methods of measuring thermal stability through temperature;

In the Scopus and Pubmed database the advanced mode was searched and in the Web of Science database the basic search was used. The criteria for collecting the data contained in the 11 eligible articles were:

A) use the alcohol test as a method of assessing milk stability;

B) have mean and values of maximum and minimum alcohol concentration in the alcohol test;

C) compare the results of stability and milk composition between the seasons (summer, autumn, winter and spring) or months of the year which may be transformed into the season in chronological form;

Systematic review (OR) results statistics

The statistical analyses of the data referring to the systematic review on the main factors affecting milk stability were performed using the R-studio software (1.3.959-1, "Middlemist Red"). The Shapiro-Wilk test (Shapiro & Wilk, 1965), based on the statistic W given by the equation [W= frac{b 2 { displaystyle sum_{i=1} n (x_{(i)}- bar{x}) 2}], was used to verify the normality of the data (H0: the data follows a Normal distribution;H1: the data does not follow a Normal distribution). Since the data did not show normality, descriptive statistical analysis was performed using data on frequency, probability, median, fashion and quartiles.

Statistics on the reanalysis of the selected articles

The statistical analyses regarding the data of the articles that evaluated the effect of the season on milk stability were performed using the statistical software Minitab (Minitab Inc., v. 19) and SAS (SAS Institute, v. 9.3). The relationships between several variables were initially accessed using scatter charts. This procedure was performed to assess the quality of the database and to observe the consistency of the data. Unusual information or patterns were reviewed in the database. However, outliers have not been removed, as they may represent the animals' responses to the most extreme treatments.

Then, descriptive statistics were obtained, as well as correlations between responses and predetermined characteristics. The comparison between the stations was performed using analysis of variance. The statistical models included the random effect of the study, in addition to the stationary effect. The homogeneity of the residues was tested by the Shapiro-Wilk test.

Linear regressions between milk stability and its composition in terms of fat, protein and lactose were initially obtained by the procedure through analysis of variance (with covariance) also considering the random effect of the study.

Results

The initial selection without duplicates had 2,774 records. After their eligibility analysis and selection by three observers, 30 selected records were obtained. However, after a request from an expert on the subject (Fischer, V.), 23 more records were included, totaling 53. In the review of the references of the 53 records, a further 31 records were added, totaling 84. On October 27, 2020, after the end of the selection of articles and their bibliographic references, a second search of bibliographic records was performed, which resulted in the insertion of three more records, totaling 87 records. (Figure 1).

The season of the year was the most frequent factor in the articles selected in the systematic review, totaling 11 articles that were eligible for statistical analysis on the effect of the season (Table 1).

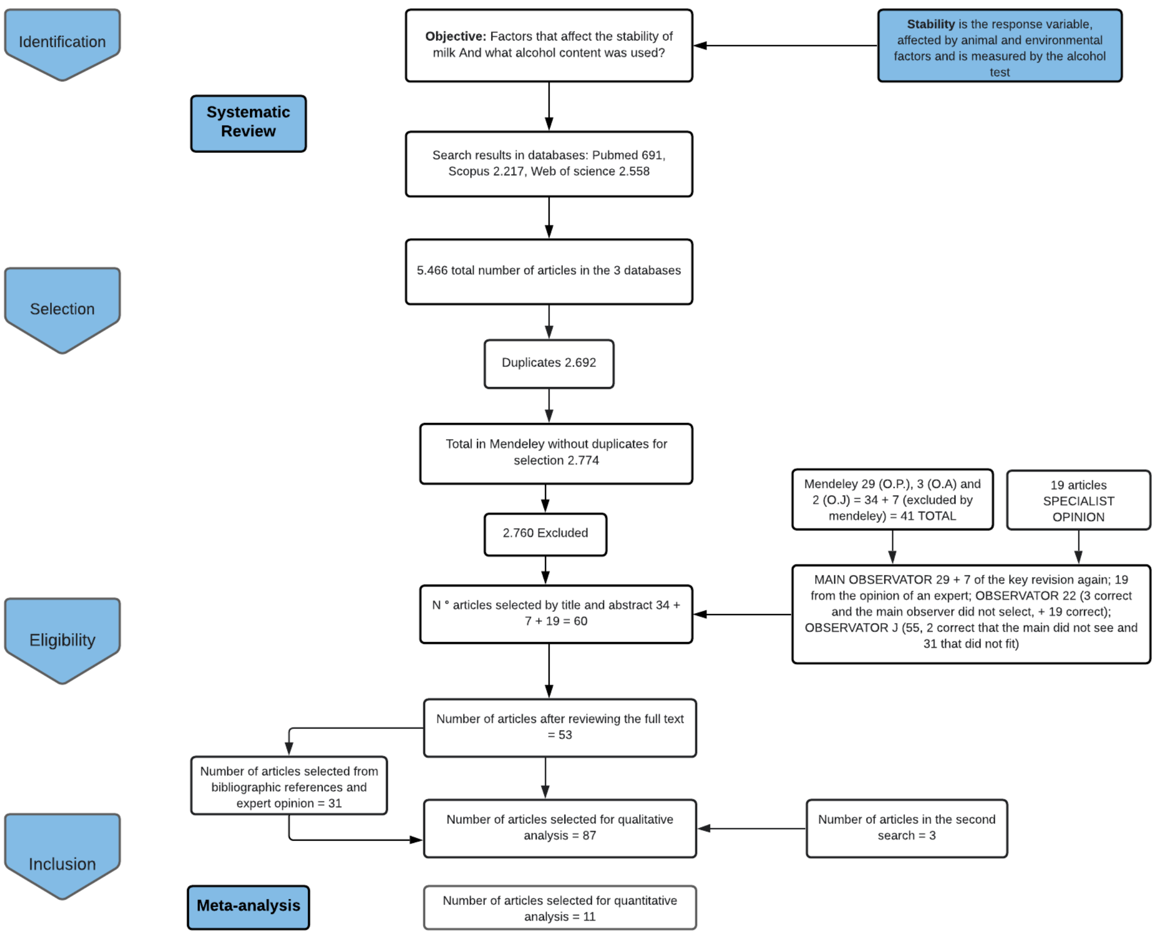

Figure 1. Diagram showing the search flow and selection of the studies.

Table 1. Articles selected by the systematic review on the seasonal effect on milk stability

Title	Author	Year	Country	Design	Test
Seasonal variation in the composition and processing characteristics of herd milk with varying proportions of milk from spring-calving and autumn-calving cows	Lin et al.	2017	Ireland	Cross-sectional study	Alcohol test and heat time
Seasonal variation, method of determination of bovine milk stability, and its relation with physical, chemical, and sanitary characteristics of raw milk	Machado et al.	2017	Brazil	Cross-sectional study	Alcohol test
Effect of seasonal variation on the composition and properties of raw milk destined for processing in the UK	Chen et al.	2014	United Kingdon	Cross-sectional study	Alcohol test
Effect of the kappa-casein gene polymorphism, breed and seasonality on physicochemical characteristics, composition and stability of bovine milk	Botaro et al.	2009	Brazil	Cross-sectional study	Alcohol test
Investigation of the effects of season, milking region, sterilization process and storage conditions on milk and UHT milk physico-chemical characteristics: a multidimensional statistical approach	Gaucher et al.	2008	French	Cross-sectional study	Alcohol test
Inestabilidad de la leche asociada a componentes lácteos y estacionalidad en vacas a pastoreo	Barchiesi et al.	2007	Chile	Cross-sectional study	Alcohol test
Occurrence of unstable to alcohol 76% and non-acid-milk and influence on physico-chemical aspects of milk	Marques et al.	2007	Brazil	Cross-sectional study	Alcohol test
Composition of milk with casein instability	Oliveira et al.	2006	Brazil	Cross-sectional study	Alcohol test
Factors affecting the ethanol stability of bovine skim milk: VII. Lactational and compositional effects	Horne	1986	United Kingdon	Cross-sectional study	Alcohol test

Relationship between ethanol stability of bovine milk and natural variations in milk composition	Donnelly et al.	1986	Umited Kingdon	Cross-sectional study	Alcohol test
The heat coagulation of milk – variations in the compositions, heat stability and other tests of milk from four cows during the course of a lactation period	Holm	1932	United States	Cross-sectional study	Heat time

Data synthesis

Data extraction was performed by transcribing the information of the original articles in an excel spreadsheet, organized according to the variables in columns containing: article code, identification of the first author, latitude and longitude, country, year of sample collection, the breed of the animals, number of animals, number of milk samples collected, time of year for sampling, minimum and maximum values of alcohol (°GL) used in the alcohol test, mean alcohol concentration (°GL) used to coagulate the samples; and concentration of total solids, protein, fat, lactose, casein, α-1,α-2, β and k-caseins, nonprotein nitrogen (NPN) values in (g/100 g) beyond calcium and phosphorus concentrations (mg/100 g) and the diameter of casein micelles (nm).

Of the 11 articles filtered by search and selection during the systematic review, only 7 articles met the selection criteria for the analysis of their data (Table 2), and the other articles did not meet the selection criteria (Table 3).

Table 2. Description of articles on the effect of the milk stability season included in the analysis

Author	N° samples	Milk type	Sample type	Season	°GL	Milk solids
Lin et al. 2017	-	Defatted raw milk	Bulk	Chronological	30 to 98	Evaluated
Machado et al. 2017	1.700	Raw	Bulk	Chronological (transformed)	68 to 90	Evaluated
Chen et al. 2014	25	Raw	Bulk	Chronological	62 to 91	Evaluated
Gaucher et al. 2008	10	LRaw	Bulk	Chronological	50 to 95	Evaluated
Oliveira et al 2006	282	Raw	Bulk	Chronological	> or < 70*	Evaluated
Horne 1986	241	Defatted raw milk	Single	Chronological	20 to 100	-
Donnelly et al.1986	-	Raw	Single	Chronological (transformed)	60 to 100	-

*In this study, only the number of coagulated samples with higher or lower degrees at 70°GL were provided, but these were not specified.

Table 3. Description of articles excluded from the analysis

Author	Season	°GL	Justification
Botaro et al. 2009	Rainy or dry	70 a 84	The evaluations were made in dry and rainy seasons in such a way that in one season it included more than 1 chronological station not susceptible to transformation
Barchiesi et al. 2007	Chronological	Only 76	The results on stability were measured in percentage of coagulated samples or not at 76°; GL; the effect of the season was concentrated on the difference in diets and the consequence of milk being stable or not at 76°GL
Marques et al. 2007	Months	Only 76	Evaluated the amount of positive and negative samples to 76°GL during the months of the year and which months showed the highest number of coagulated samples
Holm 1932	Chronological	Heat	He used the heat coagulation time test on the samples to test for stability and gave greater emphasis to the effect of lactation stages

Statistical analyses of data

We considered the data of the seasons in the chronological form of 7 articles, the averages of the alcoholic values used to coagulate the milk samples of each season supplied in 7 studies (46 n = n° of observations), the total solids contents of 5 studies (18 n), the fat values of 5 works (18 n), protein of 5 works (18 n), lactose of 5 studies (18 n), and the values of NPN, calcium, phosphorus and size of the casein micelle of only 1 study (Lin et al. 2017) (4 n).

The seasons of the year influenced the stability of milk (P<0.05). In the spring there was greater stability of milk (84.73°GL[A]) and in the autumn less stability (81.18°GL[C]), while the winter and summer ([B]) values did not differ statistically (Table 4).

Table 4. Seasonal variation in the bovine milk stability

Season	**Mean**	**SE**	***P*-value[1]**	**R^2**
Spring	84.73[A]	1.82		
Winter	82.58[B]	1.46	0.002	0.877
Summer	82.16[B]	2.26		
Autumn	81.18[C]	1.65		

[1] Season effect. Model also included the fixed effect of year (*P* = 0.693) and the random effect of study (*P* = 0.060).
[A,B,C] Values within a column with different superscripts differ significantly at P ≤ 0.05 according to Tukey's test.

By correlating milk stability and milk solids, protein, fat and lactose concentrations were positively correlated with stability (P ≤ 0.05), while casein content tended to be positively correlated with stability (0.05 > P < 0.1). The values of total solids, α1, α2 and k-casein, calcium, phosphorus and casein micelle size were negatively correlated with milk stability, these values may have tended to this behavior because they were mentioned in only one of the 7 articles considered for review, that is, there is not a sufficient degree of freedom for this result. Total casein concentration tended to be positively correlated with stability (0.05 > P <0.10). However, the milk solids content, k-casein content and phosphorus content did not correlate with stability (P ≥ 0.10) (Table 5).

Table 5. Linear correlation between bovine milk stability and the raw milk composition

Variable	Correlation	*P*-value
Total solids, %	-0.041	0.825
Fat, %	0.573	0.001
Protein, %	0.674	<0.001
Lactose, %	0.557	0.001
*Casein, %	0.574	0.051
*α1-casein, %	-0.838	0.009
*α2- casein, %	-0.990	<0.001
*β-casein, %	0.994	<0.001
*k-casein, %	-0.573	0.138
*NPN, %	0.949	<0.001
*Ca, mg/100g	-0.715	0.046
*P, mg/100g	-0.051	0.904
*Casein micelle size, nm	-0.978	<0.001

[1] Season effect. Model also included the fixed effect of year (*P* = 0.693) and the random effect of study (*P* = 0.060).
*Values present in only 1 article (Lin et al., 2017).

After analyzing the regression charts, it was observed that with the increase of 1 unit of alcohol content in the alcohol test, fat, protein and lactose values increased, respectively, by 9.9%, 4.5% and 18.1% (Figure 2). It is known that some authors have related unstable milks with lower levels of protein (Ponce and Hernández, 2005; Zanela et al., 2006; Marques et al., 2007) and fat (Oliveira and Tim, 2006; Marques et al., 2007; Zanela et al., 2009), as well as in this study. However, in contrast, lactose levels are usually higher in less stable milk (Marques et al., 2007). The quadratic adjustments of the models were also tested, although they are not presented in this work, because their terms were not significant (P>0.10).

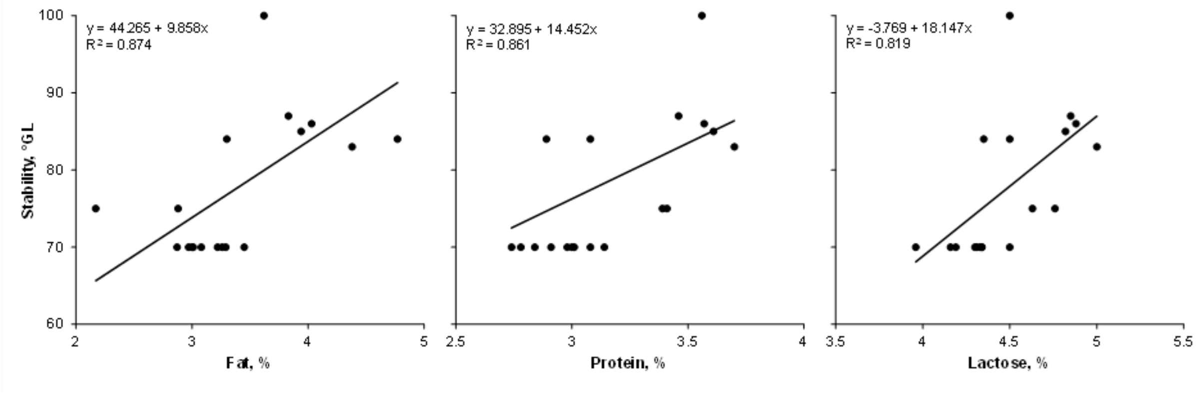

Figure 2. Equations[1] estimating the bovine milk stability from raw milk composition in terms of fat, protein, and lactose.
[1] Models also included the random effect of study (*P* = 0.09).

Discussion

The lack of compatibility and standardization in the way to collect and evaluate data on milk stability in the different seasons of the year between the articles made it impossible to achieve goal-analysis, making possible only a statistical reanalysis of the data contained in the articles filtered by the systematic review.

The effect of the season on milk stability

It is known that the season of the year differs as to the duration of the photoperiod, the temperature (Abreu et al., 2020), the available feeding (Hernandez & Ponce, 2005) and the concentration of deliveries at certain times of the year (Donnelly & Horne, 1986) resulting in different climatic and food conditions between seasons. The present study shows relatively small changes in stability between seasons, with emphasis on higher stability in spring (84.73%). This result was probably due to the favorable combination of forage availability with temperatures and more moderate solar radiation, with this may be a greater food and nutritional contribution, resulting in a higher concentration of some dairy components, such as protein (Chen et al., 2014). In winter and summer, the values were intermediate, no different from spring and autumn. It is known that in autumn and summer, the lowest stability values may have occurred, respectively, due to the reduction of forage supply and thermal stress. The lowest values of stability during the summer may have been due to higher values of solar radiation and ambient temperature (Lin et al., 2009; Abreu et al., 2020), which can cause thermal stress in animals and affect their performance by interfering with metabolism, physiology and productive performance of the animal (Roth, 2017), despite the correct supply of nutrients.

In this sense, the effect of the season may favor the occurrence of feed restrictions in systems where there is greater dependence on pastures, such as systems in southern Brazil and Ireland, and may explain the results found during autumn/winter. However, feed restriction may occur at other times of the year, depending on climatic conditions and technological input, as well as the possibility of using conserved forages. An example of this, Manske et al. (2020) observed lower milk stability in alcohol test in the late summer and early autumn months, times of lower pasture availability in southern Brazil due to lower precipitation, compared to the months of higher rainfall during the winter and early spring, in agreement with previous results reported by Marques et al. (2007) and Zanela et al. (2009). The scarcity of pastures, caused by lower rainfall, mainly in the dry season (Botaro et al., 2009) and the lower quality of pastures due to their higher fiber content (Bataglini et al., 2013) were also related to decreased in the stability of milk.

During winter, in some regions of the planet, animals are usually confined, relying on feeding systems based on higher amounts of concentrate (Chen et al., 2014) and the use of conserved forage, which explains the results on intermediate stability during the winter months. In the south of Brazil, temperate forage species such as *Avena strigosa* and *Lolium multiflorum* are available on late fall, winter and early spring, improving the supply of nutrients to the animals, which corroborates with the results of this reanalysis during the spring.

The lower stability values in summer, which are tentatively attributed to heat stress, might be explained by the negative effects of high temperature and solar radiation. When animals are exposed to temperatures above the thermal comfort range, there may be changes in cows' metabolism such as ruminal and metabolic acidosis, dehydration and increased plasma cortisol, reducing blood flow to the mammary gland and increasing the death of mammary epithelial cells (Tao et al., 2018), and as a consequence there will be a dramatic decrease in milk production and a variable change in its components (Bernabucci et al., 2010; Cowley et al., 2015; Tao et al., 2018). The severe thermal stress combined with the lack of shade increased the titratable acidity of milk (Abreu et al., 2020), which may have increased the concentration of ionic calcium in milk, an important factor that regulates milk stability (Chavez et al., 2004; Horne, 2015). Our results are partially in agreement with those of Lin et al. (2009) who reported lower stability during the summer.

The effect of milk stability on milk solids

The concentration of dairy components may be affected by food (Bataglini et al., 2013), but also by animal genetics (Botaro et al., 2009). The supply of nutrients (amino acids and energy, in addition to minerals) is positively related to the greater synthesis of milk protein (Gabbi et al., 2018).

The positive correlation between lactose concentration and stability is supported by previous studies, which show higher lactose concentrations in stable milk compared to unstable milk (Marques et al., 2007; Fruscalso et al., 2013; Stumpf et al., 2013). According to Fagnani et al. (2017), the higher the lactose percentage, the lower the chances of low milk stability. The osmotic balance of the secretory epithelium is determined by lactose and diffusible ions (Kuhn et al., 1980), and there is usually an inverse relationship between the lactose concentration and that of milk minerals (Rook & Wood, 1954). As a result, Na^{+} and Cl^{+} concentrations increase, while lactose decreases, both via the paracellular pathway (Fagnani et al., 2017). Knowing that Na^{+}, Cl^{+} and K^{+2} promote the destabilization of casein micelle by interfering with its ionic strength (Chavez et al., 2004), if the lactose levels are lower in unstable milks, there is a higher concentration of Na^{+}, Cl^{+} and K^{+2} ions and, thereforelowering milk stability.

A further relationship of lactose concentration with milk stability would be in relation to the arterial supply of glucose (Wang et al., 2016), which may be affected by the already mentioned feed restriction, depriving the supply of glucose to the mammary gland - it has already been associated with alcoholic milk instability (Zanela et al., 2006).

The positive correlation between milk stability and milk protein concentration evidenced in the present study may be explained by the positive effect of nutrient supply that supports protein synthesis (Gabbi et al., 2018), which provide more substrate for the Krebs cycle, increasing citrate synthesis, and helping to maintain the integrity of the mammary tight junctions, explaining the higher values of both variables (Stumpf et al., 2013).

Other studies corroborate the positive effect of the nutritional contribution on milk stability and protein concentration. Although the studies filtered in the present study do not present informations about the diet offered to the animals, when the availability of pasture increased and/or if supplementation with concentrate was provided, higher protein concentration and milk stability values were observed (O'Brien et al. 1999). The greater digestibility of the pastures combined with structural characteristics favoring the apprehension of the pasture such as pasture mass and pasture height increase the citrate concentration (Dunshea et al., 2019). Citrate is recognized as a stabilizing agent by reducing the concentration and ionic calcium of milk (Santos & Fonseca, 2007).

The positive correlation between stability and fat in this reanalysis is not in agreement with Chen et al. (2014) (-0,488). According to Fagnani et al. (2014), the concentration of fat is one of the most variable components. In addition to seasonality, other factors influence it, such as breed, diet and lactation stage (Ordoñez, 2005). The increment in nutrient supply increased the synthesis of milk components (Gabbi et al., 2018).).

Limitations of studies for analysis

With the low sample number (n) retained in the analyses due to the lack of standardization in the collection and evaluation of data of the 11 studies and, knowing the variability of factors that influence the correlation between seasons and milk stability, it is difficult to accurately state the effect of the station on stability. Moreover, the differences in the ways of evaluating milk stability by alcohol testinghindered the analysis.

Conclusion

During the spring, milk is more stable compared with other season what isassociated with better quality and availability of pastures, the greater nutritional supply allied to more favorable weather conditions for animal welfare. There is a positive association between stability and protein, fat and lactose concentrations.

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CAPÍTULO IV

The effect of diet restriction on raw milk stability - A meta-analytic approach

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Abstract

Milk stability is affected by several factors, including feed availability and quality both of them affecting nutrient availability. The objective of this study was to identify, critically evaluate and summarize the available information in the literature, through a systematic review and subsequent meta-analysis (joint data analysis) on the effect of different levels (20 to 50%) of feed restriction, on the stability of raw bovine milk. Of the 9 articles that addressed the subject filtered by the systematic review, 7 studies were included to perform the meta-analysis, With 404 observations. feed restrictionReduction of 20%, 30%, 40% and 50% of dry matter in the diet diminished ($P<0.01$) milk production (-18%), milk stability (-5%), acidity (-4%), as well protein (-3%) and lactose (-2%) concentrations, but feed restriction did not affect the values of pH, density, fat concentration, total solids and SCC. The correlation between milk production and stability was positive, higher in the control group ($r=0.30$) in relation to the restriction group ($r=0.15$). The samples of the control diet presented greater stability to the alcohol test (76.51%) compared to the samples of the restrictive diet (72.77%). This decrease by up to 4 percentage units due to restrictions of 20 to 50 % on diet intake may increase the milk rejection by dairy industry.

Key Words: alcohol test, meta-analysis, milk stability, raw milk, feed restriction.

Introduction

It is known that the thermal stability of milk decreases with reduced availability of pasture (O'Brien et al., 1999) or diet (Zanela et al., 2006; Zanela et al. 2009; Bataglini et al., 2013; Fruscalso et al., 2013; Stumpf et al., 2013, Gabbi et al., 2015) as well with lower supply of nutrients or imbalance between nutrients such as energy and protein (Marques et al., 2010;; Gabbi et al., 2015), an increase in the fiber content of pastures and forages used in food (Barchiesi et al., 2007) and reduced consumption due to heat stress (Abreu et al., 2020).

However, the effect of feed restriction on milk stability depends, among other factors, on its severity and duration (Gabbi et al., 2015). However, there are studies that have subjected the animals to the restriction of 40% of the diet and did not observe any effect on milk stability (Barbosa et al., 2012). In addition to the supply of food and/or nutrients, factors linked to animals can influence these results, such as the lactation stage, where supplementation with alfalfa hay and concentrate did not improve the stability of cows' milk at the final lactation stage, with DEL greater than 300 days (Marques et al., 2010), and in cows with DEL greater than 230 days (Barbosa et al., 2012). Other studies evidenced that milk stability is low (< 72°GL) in the first two weeks *postpartum*, probably caused by the composition of the protein fraction of milk as well by the high acidity and concentration of Ca^{2+} at the beginning of lactation (Tsioulpas et al., 2007; Heisler et al., 2017).

The term meta-analysis was coined in 1976 by Gene Glass to report the statistical combination of independent research results, applied in a quantitative approach, but it was only in 1985 that Stern and Harris presented the qualitative version of the meta-analysis (Glass, 1976; Beaucher & Jutras, 2007). That is, it is a statistical method to aggregate the results of two or more independent studies, on the same research question, combining their results in a summary measure. They are usually performed after a systematic review.

The objective of this study was to identify, critically evaluate and summarize the available information in the literature, through a systematic review and subsequent meta-analysis (joint data analysis) on the effect of different levels of feed restriction, 20 to 50% restriction on the stability of raw bovine milk.

Material and methodology

The present manuscript is the result of a systematic review that gave rise to another article, so the initial methodology of searching and filtering articles (systematic review of the literature) is the same for both, thus being the basis for the elaboration of both.

Research question

This study identified the effect of feed restriction on the thermal stability of bovine milk to alcohol testing. This joint analysis of raw data is the result of a systematic review, whose objective was to evaluate the main factors identified as a cause of decrease in the stability of raw bovine milk. After the statistical analysis, it was found that the second most frequent factor (7.1%) that negatively affects the thermal stability of raw milk was the effect of the feeding restriction of the animals.

Research methods to identify studies

The search strategy in the literature was defined based on the main concepts in terms of PIC'o: population (P), intervention (I) and context (C). Then, the PIC'o of the following study resulted in: POPULATION (population) - milk, being samples of individual animals and/or whole milk, on the farm; INTERVENTION (intervention/interest) - related to milk stability, to LINA, to alcohol or alizarol testing, heat; CONTEXT (context) - the factors that have already been mentioned that can negatively affect milk stability. Subsequently, the search key was elaborated, and consisted of the following sequence: ("milk") AND ("LINA" OR "unstable" OR "Stability" OR "Ethanol" OR "Alcohol" OR "non Acid" OR "Heat") AND ("feed restriction" OR "thermal stress" OR "heat stress" OR "lactation stage" OR "metabolic disease" OR "metabolic disturb" OR "digestive disease" OR “acidosis” OR “mastitis” OR “agitation” OR “storage” OR “transport”).

The search was carried out on July 17, 2020, in 3 databases: PubMed, Scopus and Web of Science. A second search was carried out, with the same key, in the three databases on October 27, 2020, after the end of the selection of the articles and bibliographic references thereof, to verify if any other articles that could contribute to the systematic review had been published. The acronym TS was inserted into the Scopus and Web of Science database to find the keywords in the title, Abstract and keywords of the articles. No filtering measures were used in the databases. All records were selected and downloaded to the desktop-based Mendeley® reference storage software. The information contained in the results were title, authors, year, publication vehicle, DOI, Abstract and keywords.

Criteria for the selection of studies

With the a priori withdrawal of the duplicate articles, the eligibility of the articles was initiated, proceeding the evaluation of the fair summaries independently by three observers. Abstracts were selected for subsequent inspection if they met the criteria:

A) scientific articles and/or short papers published in journals;

B) have access to the full text;

C) have worked with raw bovine milk and sampled individually per animal;

D) contain the word "stability" related to raw milk;

E) have worked with alcohol testing and alcohol graduations or thermal stability by heating and clotting time;

F) have used methods of measuring thermal stability through temperature.

In the Scopus and PubMed database the advanced mode was searched and on the Web of Science database the basic search was used. The initial selection without duplicates had 2,774 records. After their eligibility analysis and selection by three observers, 30 selected records were obtained. However, after a request from an expert on the subject, 23 more records were included, totaling 53. In the review of the references of the 53 records, a further 31 records were added, totaling 84. On October 27, 2020, after the end of the selection of articles and their bibliographic references, a second search of bibliographic records was performed, which resulted in the insertion of three more records, totaling 87 records considered for statistical analysis.

Systematic review results statistics

Statistical analyses were performed using R-studio software (1.3.959-1, "Middlemist Red"). The Shapiro-Wilk test (Shapiro & Wilk, 1965), based on the statistic W given by the equation [W= frac{b 2 { displaystyle sum_{i=1} n (x_{(i)}-bar{x}) 2}], was used to verify the normality of the data (H0: the data follows a Normal distribution; H1: the data does not follow a Normal distribution). Since the data did not show normality, descriptive statistical analysis was performed using data on frequency, probability, median, fashion and quartiles. The feeding restriction of animals was the second most studied factor in articles that address factors that affect milk stability (7.1%), totaling 9 studies on this factor.

Data synthesis

The criteria for collecting the data contained in the 9 eligible articles were:

A) use the alcohol test as a method of assessing milk stability;

B) Measure different levels of animal feed restriction (restricted diet) and compare a diet that met 100% of the animals' dietary requirements (control diet) through the supply of dry matter per animal;

C) Possibility of access to raw data of articles.

Data extraction was performed by transcribing the information of the original articles in an Excel spreadsheet, organized, according to variables, in columns containing: article code, identification of the first author, temperature, breed of animals, number of animals, lactation days (DEL), time of year of experiment, type of system (confined or semi-self-contained), type of feeding of animals (whether silage, hay, pre-dried, feed or concentrate), amount of dry matter (MS) received by animals in both treatments (restrictive diet or control diet with 100% of nutritional requirements), number of days of adaptation (pre-restriction), number of days in restriction, number of days of recovery (post-restriction), mean of stability (°GL) used in the alcohol test to coagulate milk samples, Somatic Cell Count (SCC) and percentages of milk solids (dairy components).

Raw data joint analysis statistics (meta-analysis)

The objective of this analysis was to evaluate the relationship between production, quality and composition of milk from cows without feed restriction or animals challenged by feed restriction, quantify and model the effect of feed restriction on production, quality and composition and also evaluate the interactions between variations in milk production, quality and composition as an effect of feed restriction.

Statistical encodings, calculations and analyses

Codes with qualitative clustering criteria were used in the analytical models. In this item, the main codes were applied to characterize feeding (for example, control or restricted feeding). Other codes were used to consider the variability between all the compiled experiments (for example, the effect of the study or trial).

Performance results were evaluated as raw data or as relativized information. In the latter, the responses of the restricted treatments were relativized to the average observed in the respective control treatment, expressed as percentage of variation between treatments. The relativized responses were called 'variation' (Δ) and can be interpreted as the "constraint effect on each performance response". The calculation of Δ was adopted because it considerably reduces the effect of heterogeneity (between experiments) on the database. Statistical analyses were performed using Minitab (Minitab for Windows, v. 20). The relationships between several predetermined variables were accessed using scatter charts. This procedure was performed to assess the quality of the database and to observe the consistency of the data. Unusual information or patterns were reviewed in the database.

Descriptive analysis and linear correlations (Pearson) were performed between the physical and chemical variables of milk (protein, fat, lactose, pH, titratable acidity, alcohol stability, cryoscopy and SCC). The results of the linear correlations were plotted in graphs to illustrate the relationships between milk stability of the groups according to diet: restricted (red circles) and control (blue circles) with pH, acidity, density, cryoscopy and CCS, and protein concentrations, total fat, lactose and solids.

Variables on solids and milk stability relative to the control and challenge groups were compared by variance analysis. The random study effect and fixed treatment effect were considered in all statistical models. In addition, the effects of the production system, genetic type, temperature and body condition score were tested for all responses, but maintained in the models only when $P < 0.10$. The interactions between the groups and other factors retained in the previous stage were tested, but no factor was maintained in the model, because no significant interaction was obtained ($P < 0.10$). The residues were tested for normality using the Ryan Joiner test for the final model. Interpretation of the treatment effect was performed in 5 and 10%. The regression adjustment between the variation (Δ, % in relation to the control group) of milk production, quality and composition and the increase of feed restriction levels (%) was evaluated using linear and quadratic models. Only the responses in which the effect of dietary restriction was significant ($P < 0.10$) were used in the analysis of variance. The studies were considered as a random effect in all analyses.

Results

After analyzing the nine articles (Table 1), seven articles werefeed restrictionas they met the selection criteria. The two excluded articles failed to meet the selection criteria, the most critical being the non-use of treatments that compared the effect on animals of a complete diet versus a restrictive diet (Table 2).

Table 1. Articles selected by the systematic review

Title	Author	Restriction level	Access to raw data
Effect of reducing daily herbage allowance during early lactation on composition and processing characteristics of milk from spring-calved herds	Gulati et al. 2019	Restricted amount of supplement	No
Behaviors associated with cows more prone to produce milk with reduced stability to ethanol test due to feeding restriction	Stumpf et al. 2016	50% of the diet	Yes
Milk traits of lactating cows submitted to feed restriction	Gabbi et al. 2015	40 to 50% of the diet	Yes
Feeding restriction impairs milk yield and physicochemical properties rendering it less suitable for sale.	Fruscalso et al. 2013	50% of the diet	Yes
Severe feed restriction increases permeability of mammary gland cell tight junctions and reduces ethanol stability of milk	Stumpf et al. 2013	50% of the diet	Yes
Electrophoretic characterization of proteins and milk stability of cows submitted to feeding restriction	Barbosa et al. 2012	40% of the diet 30% nutrients	Yes
Unstable nonacid milk and milk composition of Jersey cows on feed restriction	Zanela et al. 2006	40% of the diet	Yes
Effect of three types of diet on the appearance of metabolic disorders and their relationship with changes in milk composition in Holstein Friesian cows	Hernandéz et al. 2005	20 and 50% of the diet	No
Milk instability as a result of an increase calcium ion content	Seekles et al. 1952	Did not evaluate only used argument for discussion of results	-

Table 2. Evaluation of articles that did not meet the criteria for analysis

Author	Restriction levels	Justification
Gulati et al. 2019	Restricted supplementation no nutritional requirements via diet	We did not perform the alcohol test to evaluate the stability but the coagulation time test to heat and we did not obtain access to the raw data.
Seekles et al. 1952	Did not present an experiment evaluating restriction levels	He only used the feed restriction factor to explain that during the period of the Second World War thousands of animals in France were under periods of feed restriction and this may be a cause of decreased milk stability, and we didn't get access to the raw data either.

However, it was noticed that the number of data available within the seven articles was very low, so it was decided to contact the authors and request the individual data of the animals in the experiments, in addition to including two studies in the "gray literature" (doctoral thesis still not published in an editorial journal) feed restriction(Table 3). Thus, the present article has individual data from seven papers that fit the selection criteria (Figure 1).

Table 3. Characteristics of articles included in the joint raw data analysis

Origin	Type of study	Author and year	Level of feed restriction*	N° of comments (n)
Expert opinion	Dissertation	Schmidt (2015)	50%	71
Systematic review	Article	Gabbi et al. 2013	30%	83
Systematic review	Article	Stumpf et al. 2013	50%	24
Systematic review	Article	Fruscalso et al., 2013	50%	59
Systematic review	Article	Barbosa et al., 2012	40%	31
Systematic review	Article	Zanela et al., 2006	40%	69
Expert opinion	Experiment	Zanela M.B. 2006	20%	59

*Percentage of diet decrease date to animals represented in dry matter (DM)

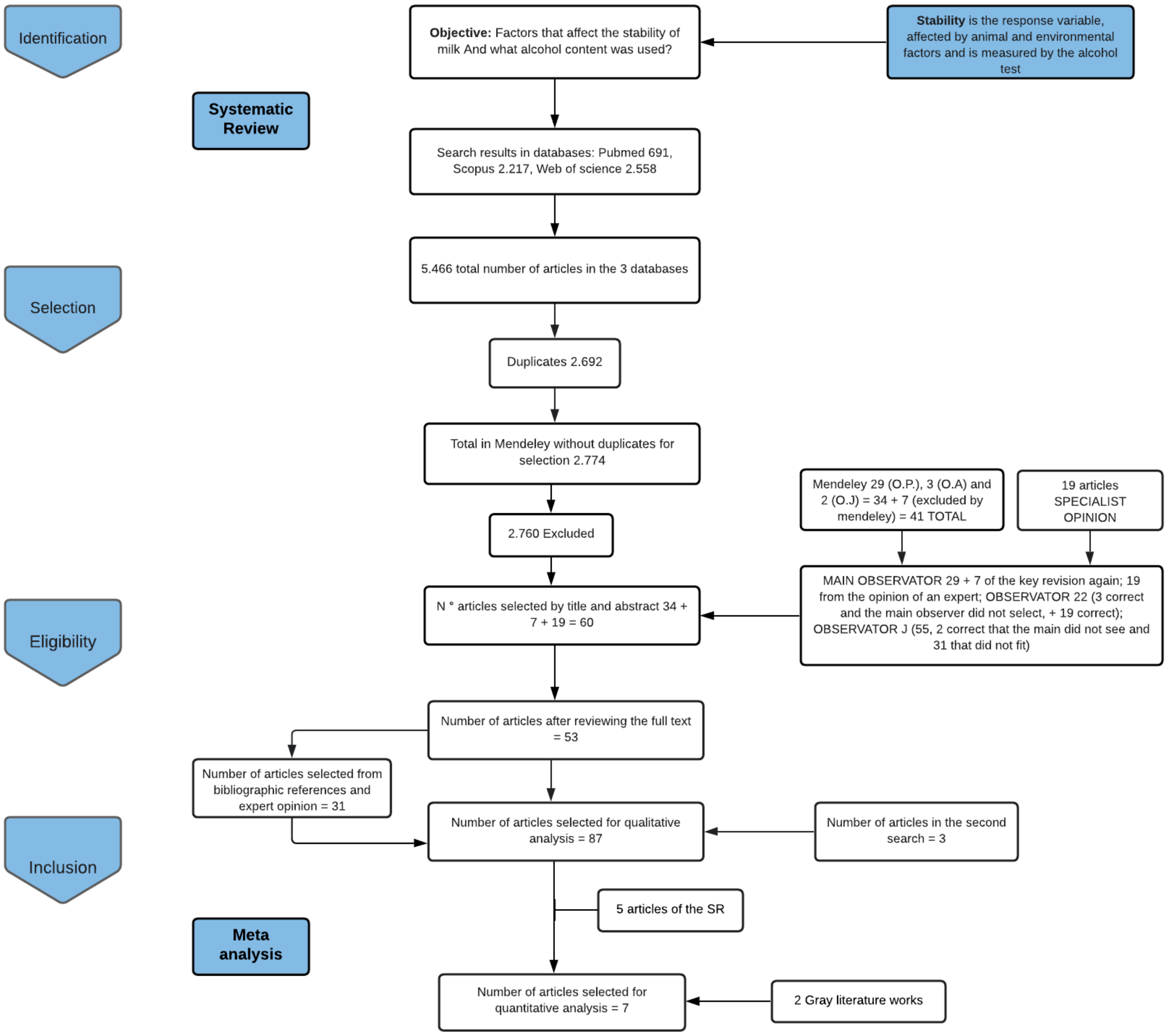

Figure 1. Diagram showing the search flow and selection of the studies.

Raw data joint analysis statistics (meta-analysis)

Database description

After extraction of the data contained in the 7 studies, descriptive statistics were performed with a significant sample number (n = 404) that enabled the analyses, with the variables common to all studies, together with the values of the quartiles in the analysis.

Effect of feeding restriction

When observing the correlations (Figure 2); (in red positive correlations, in blue, negative), it is verified that the correlation between milk production and stability was positive, but higher in the control group (r=0.30) in relation to the restriction group (r=0.15). Acidity was highly correlated with feed restriction, going from negative (-0.09) in the control group to positive (0.14) in the group that suffered restriction. The correlation between milk solids and protein and fat content was maintained in both treatments (r=0.74, r=0.92; r=0.75, r=0.91), respectively.

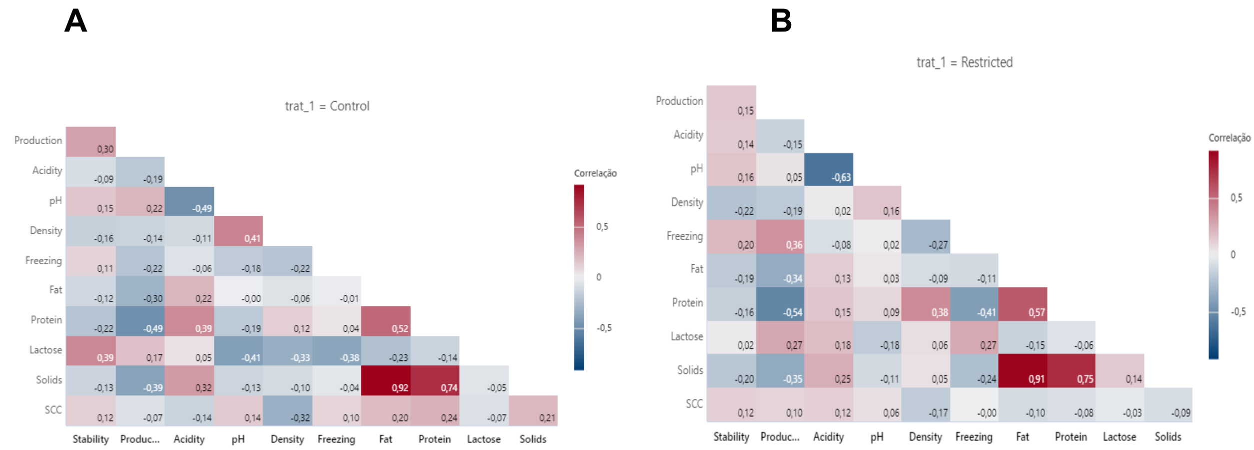

Figure 2. Correlations among milk production, quality and composition in control (A) and feed restriction group (B)

Different levels of dietary restriction (reduction of 20%, 30%, 40% and 50% of dry matter in the diet) reduced ($P<0.01$) milk production (-18%), ethanol stability (-5%) and acidity (-4%), as well protein (-3%) and lactose (-2%) concentrations, but did not affect pH values, density, fat concentration, total solids and SCC (Table 4). The milk samples from animals receiving the control diet presented higher stability to the alcohol test compared to the restrictive diet samples (Table 4), which is corroborated by the values of the correlation coefficients (Figure 4), that is, when the animals receive the control diet, the correlations are positive, such as milk stability and production; stability and lactose.

Table 4. Effects of feed restriction on milk production, quality, and composition

Variables	Treatments		Δ, %	RSE[1]	*P*-value [2]	Model [3]	Part. [4]
	Control	Restricted					
Milk production, kg/day	14.51	11.85	-18.3	4.13	<0.001	G	12.1
Stability, % ethanol	76.51	72.77	-4.9	4.15	<0.001	-	22.7
Acidity	16.69	16.01	-4.1	1.57	0.001	B	15.7
pH	6.72	6.74	0.3	0.17	0.151	-	0.3
Density, g/L	1030.1	1029.9	0.0	1.32	0.427	S, T	0.0
Freezing temperature, °C*	-0.543	-0.540	0.6	0.01	0.191	-	1.3
Fat content, %	4.41	4.42	0.2	0.90	0.980	G	0.0
Protein content, %	3.48	3.36	-3.4	0.35	0.005	G	11.8
Lactose content, %	4.43	4.36	-1.6	0.25	0.016	G	12.2
Total solids, %	13.20	13.06	-1.1	1.25	0.280	G	0.5
Somatic cell count, n° x 1000 cells/ml	353.6	280.7	-20.6	350	0.243	-	0.6

[1] Residual standard deviation.
[2] *P*-value indicates the probability of feeding restriction effect.
[3] The random effect of studies was considered ($P < 0.10$) for all responses. The effects of production system (S), genetic type (G), temperature (T), and body condition score (B) were tested, but maintained in the models only when significant ($P < 0.10$). No significant interaction was found ($P > 0.10$).
[4] Partition of total variance attributed to feeding restriction effect.
* -0.512 ºC a -0.536 ºC - (Brasil, 2018).

Milk components negatively affected by feed restriction were milk production in 72% of comparisons; milk stability in 77% of comparisons; milk acidity in 66% of comparisons; protein content in 74% of comparisons and lactose content in 64% of comparisons (Figures 3, 4, 5, 6, 7). The other characteristics of the milk were not influenced by the diet restriction. The regression analysis reveals that each percentage point of feed restriction imposed on animals there were significant reductions of 0.4% in milk production (Figure 3); of 0.13% in milk stability (Figure 4), of 0.11% in milk acidity (Figure 5), 0.14% in protein content (Figure 6) and 0.06% in lactose content (Figure 7). In the models presented (Figures 3 to 7), the dietary restriction negatively affected the production, stability, titratable acidity, protein content and lactose content of milk in more than 64% of the comparisons.

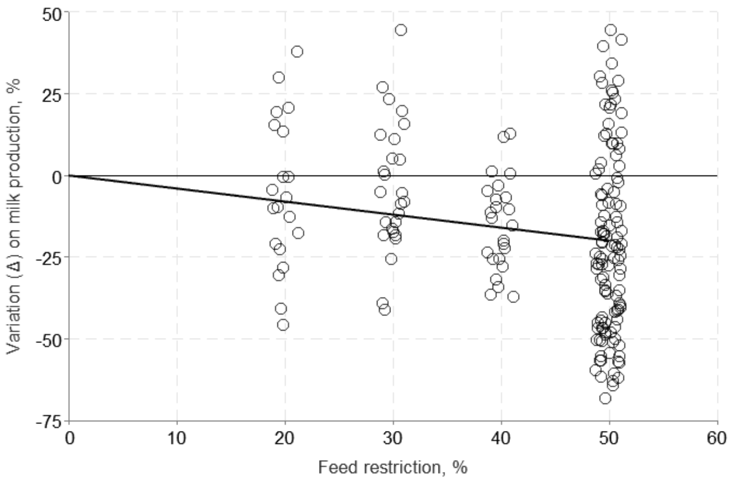

Figure 3. Modeling the variation (Δ) on milk production as an effect of feed restriction[1,2]

[1] Model: $y = -0.3994x$ $y = -0.3994x$; where y y is the variation (Δ) and x x is the feed restriction, both expressed as %.
The random effect of studies was considered (*P* < 0.10) and the R^2 was 0.36.
Quadratic term was tested, but it is not presented due to the lack of significance (*P* > 0.10).
[2] Reduction (Δ<0) was observed in 72% of the comparisons.

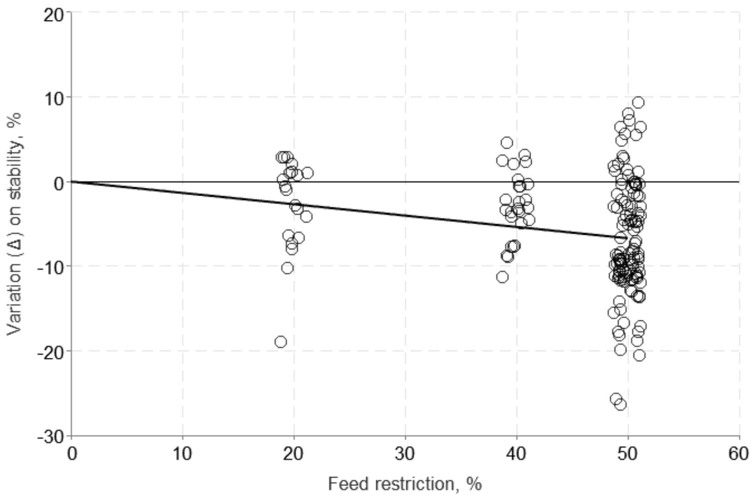

Figure 4. Modeling the variation (Δ) on milk stability as an effect of feed restriction[1,2]

[1] Model: $y = -0.1341x$ $y = -0.1341x$; where y y is the variation (Δ) and x x is the feed restriction, both expressed as %.
The random effect of studies was considered (*P* < 0.10) and the R^2 was 0.58.
Quadratic term was tested, but it is not presented due to the lack of significance (*P* > 0.10).
[2] Reduction (Δ<0) was observed in 77% of the comparisons.

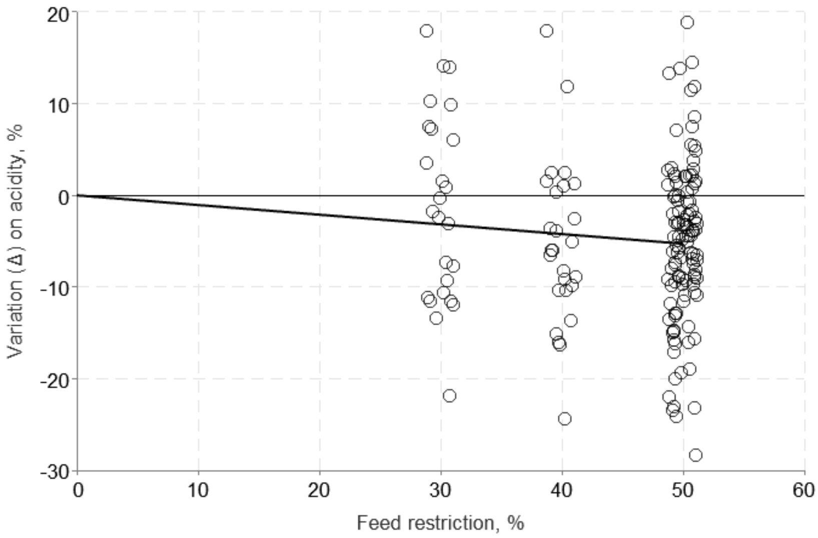

Figure 5. Modeling the variation (Δ) on milk acidity as an effect of feed restriction[1,2]

[1] Model: $y = -0.1056x$ $y = -0.1056x$; where y y is the variation (Δ) and x x is the feed restriction, both expressed as %.
The random effect of studies was considered (*P* < 0.10) and the R^2 was 0.28.
Quadratic term was tested, but it is not presented due to the lack of significance (*P* > 0.10).
[2] Reduction (Δ<0) was observed in 66% of the comparisons.
Acidity = g ácido lático/100 mL

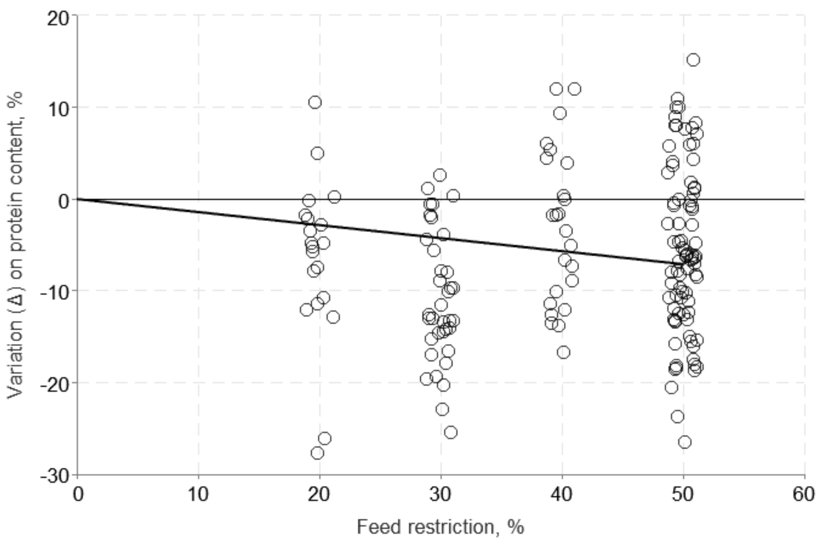

Figure 6. Modeling the variation (Δ) on milk protein content as an effect of feed restriction[1,2]

[1] Model: $y = -0.1420x$ $y = -0.1420x$; where y y is the variation (Δ) and x x is the feed restriction, both expressed as %.
The random effect of studies was considered (*P* < 0.10) and the R^2 was 0.39.
Quadratic term was tested, but it is not presented due to the lack of significance (*P* > 0.10).
[2] Reduction (Δ<0) was observed in 74% of the comparisons.

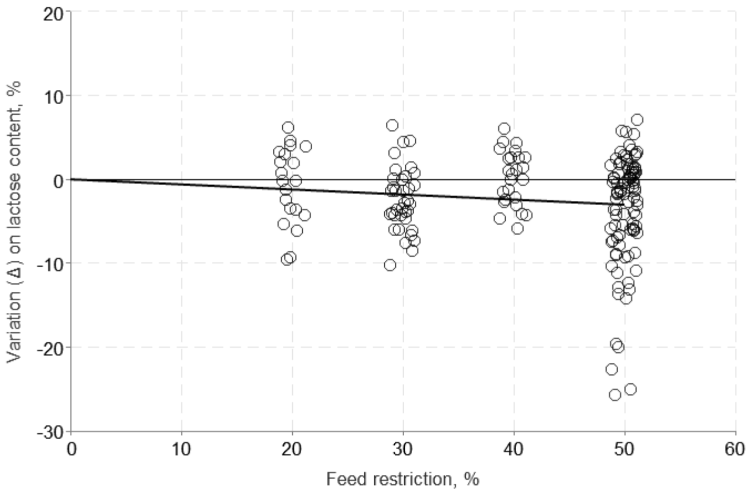

Figure 7. Modeling the variation (Δ) on milk lactose content as an effect of feed restriction[1,2]

[1] Model: $y = -0.0605x$ $y = -0.0605x$; where y y is the variation (Δ) and x x is the feed restriction, both expressed as %.
The random effect of studies was considered (*P* < 0.10) and the R^2 was 0.27.
Quadratic term was tested, but it is not presented due to the lack of significance (*P* > 0.10).
[2] Reduction (Δ<0) was observed in 64% of the comparisons.

Discussion

Quality analysis of studies

The systematic review of the literature showed that feed restriction is the second factor most related to low milk stability. The inspection of the 9 selected articles on the subject revealed difficulties to compare the results, such as differences in the magnitude of restriction, lactational stage, food management, expression of stability, resulting in a small sample number, which would make it impossible to perform the meta-analysis. However the use of raw data provided by authors, it was possible to perform a meta-analysis to re-analyze the data, thus verifying, with a higher number of observations (n = 404) the effect of the feed restriction on milk stability and other milk solids.

Sellected studies differed in the feed offered to cows, from complete diet, composed of silage and concentrated at the trough and access to pastures (Zanela et al., 2006) or silage, concentrate and hay at the trough (Barbosa et al., 2012), grazing and concentrate supplement (Fruscalso et al., 2013). Regarding feed restriction, we decided to calculate the amount of dry matter that animals received during the control diet and the restrictive diet, since some studies restricting them chose to reduce energy or protein only in the restriction (Schmidt, 2015), resctrict of the whole diet or remove only the concentrate from the restrictive diet (Zanela, 2006). In relation to what was previously published by Gabbi et al (2015), the present study increased the levels of feed restriction from 0, 40 and 50% to 0, 30, 40 and 50%.

Statistics of the joint analysis of the raw data

Based on the individual data of the animals used in these experiments, the reanalysis of all the data together confirmed and quantified the deleterious effect of the feed restriction on milk stability, so that the increase of each percentage point of feed restriction imposed on animals reduced milk stability by 0.13% (Figure 6). In addition, the negative effect of feed restriction on milk production, acidity, and protein and lactose concentrations were confirmed, without changing the pH, density, fat concentration, total solids and SCC values (Figure 4).

The lower production of milk caused by feed restriction (Figure 5) is due to the lower intake of nutrients and consequent uptake by the mammary gland, since the reduction of consumption promotes the reduction of blood flow (Guinard-Flament et al., 2006, Guinard-Flament et al., 2007). The reduction in milk production was observed in all studies that restricted food supply (Ponce & Hernández, 2001; Zanela et al., 2006; Barbosa et al., 2012; Fruscalso et al., 2013; Stumpf et al., 2013; Gulati et al. 2019).

The relationship between the reduction of milk stability and feed restriction (Figure 6) may be explained by the stress caused by the sudden restriction of food supply and/or nutritional imbalance (Verkerk et al., 1998), altering animal behavior, especially increasing competition events and discomfort manifestations such as vocalizations, agonal behavior, stereotypes, resulting in increased cortisol secretion (Stumpf et al., 2013). These stress-induced behavioral and metabolic changes were related to increased permeability of tight junction mammary epithelial cells (Stelwagen et al., 2000), increasing plasma lactose concentration, reducing lactose concentration in milk, but increasing the sodium content in milk (Stumpf et al., 2013).. Moreover, reduction in nutritional levels, mainly in relation to total digestible nutrient levels decrease protein synthesis (Gabbi et al., 2018), especially of κ-casein, that is largely responsible for the stability of casein micelle or its glycosylation (Martins et al., 2019) and consequently the stability of the milk might be reduced.

The reduction in protein (Figure 8) and lactose (Figure 9) concentrations of milk, as a function of the magnitude of the feed restriction, is related to the reduction of nutritional intake, resulting in lower absorption of nutrients, especially glucose, by the mammary gland, with less protein and lactose synthesis (Guinard-Flament et al., 2006, Guinard-Flament et al., 2007). On the other hand, the reduction of acidity (Figure 7) as a function of the magnitude of the restriction may be related to the reduction of the protein concentration, since together with minerals such as phosphates and dissolved gasses confer natural acidity of milk (Schmidt et al., 1996). In the case of unbalanced diets with excess protein in relation to energy, the lowest stability observed (Marques et al., 2010; Schmidt, 2015) may be related to the excess of degradable protein, which, in turn, was related to the lower glycosylation of κ-casein, reducing milk stability, as verified by Martins et al. (2019).

All studies included in this meta-analysis adapted the animals to a diet similar to the control treatment, during a period that ranged from 7 to 17 days according to the study, before reducing the food supply, which probably evidenced the differences between the groups after the abrupt decrease of the diet offer in levels of 30 to 50%. However, in general, all correlations involving milk stability obtained low values, this shows that stability is not a factor affected only by feed restriction but a multifactorial problem, which may have had the effect of other variables not considered in the studies addressed here.

Conclusion

Feed restriction reduces milk production, lactose and protein concentrations, as well as stability in an increasing way with its severity. Milk stability in alcohol testing decreased by up to 4 percentage units with dietary restrictions of 20-50%, which can cause milk rejection by industry.

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CONSIDERAÇÕES FINAIS

Sabe-se que a estabilidade do leite cru bovino é afetada por muitos fatores. Após realizar uma revisão sistemática da literatura, verificamos que 35 fatores podem afetar a estabilidade do leite, diminuindo-a ao teste do álcool e do tempo de coagulação ao calor. Entre os principais fatores mencionados, a estação do ano, que foi relacionada a outros fatores, como a disponibilidade de alimentos e o estresse térmico, e a restrição alimentar, que por sua vez também pode sofrer interferência da estação do ano, foram os dois fatores mais mencionados, 9,5% e 7,9%, respectivamente. Ambos os fatores mais mencionados são situações corriqueiras nas fazendas, principalmente nas brasileiras.

O interesse científico em entender e elucidar as causas da baixa estabilidade do leite se mostram ainda relevantes durante uma faixa de pouco mais de um século, desde as primeiras publicações, evidenciam que tal fenômeno causa perdas e prejuízos econômicos e até mesmo sociais, já que um leite com baixa estabilidade, normalmente não é utilizado pela indústria, que o descarta. Tal problema foi abordado por autores de 25 países diferentes só corrobora para mostrar a importância da baixa estabilidade do leite para esses países, sendo muitos dependentes da produção de leite UHT, o produto que mais exige estabilidade do leite para que o processamento térmico ocorra de maneira correta e não cause sedimentação do produto nos equipamentos, consequentemente, menor eficiência produtiva. Contudo, é equivocada a postura de nos balizarmos em cima da variável estabilidade do leite para avaliarmos a qualidade do mesmo.

Em relação ao teste do álcool, sabe-se que é um teste sensível e que fatores ligados ao animal e a composição do leite podem causar um resultado positivo ao teste, como por exemplo leites de animais no final da lactação, ou de um tecido mamário ligeiramente irritado e inflamado, com quantidades maiores de CCS, leites com um desbalanço de sais, com excesso de cálcio ou magnésio, ou seja, esses leites são produtos de qualidade, porém por causa desses fatores acabam apontando baixa estabilidade. Após o levantamento feito pela revisão da literatura, diversos autores encontraram leites de boa qualidade, com baixos teores de CPP, CCS, acidez normal e altos teores de sólidos do leite, porém que deram positivo no teste do álcool. o que confirma que o mesmo não é totalmente confiável para que a indústria o utilize como parâmetro de qualidade do produto, ocorrendo um superdimensionamento do teste do álcool e acarretando no não aproveitamento de leites de qualidade pela indústria. Outro ponto, é o fato de que muitos leites com baixa estabilidade apresentaram altos teores de proteína e suas diferentes frações, característica desejada para a produção de queijos, direcionando para um possível uso dos leites com baixa estabilidade.

A questão relacionando as graduações utilizadas mostra que há uma grande influência das graduações superiores usadas em experimentos científicos, que querem confrontar a resistência da matéria prima a graduação máxima de álcool no teste, mas também há graduações mínimas de valores baixos pontuando a falta de uma medida padronizada e a influência de trabalhos de países em que se trabalha com graduações inferiores por causa do acesso deficiente a métodos de refrigeração adequados e problemas de qualidade do leite, impossibilitando o uso de graduações maiores. Diante desse cenário, ainda temos a necessidade de buscar novas tecnologias, baratas e rápidas, de testar a estabilidade do leite, mas também de diferenciar o leite com baixa estabilidade (LINA), que pode ser aproveitado pela indústria para transformações em outros produtos lácteos diferentes do leite UHT, do leite ácido, que decorre da alta quantidade de microorganismos acidificantes e tal condição compromete as características biológicas, físicas e químicas para o seu aproveitamento pela indústria. Uma padronização mundial sobre a graduação adequada ao teste do álcool, para verificar a estabilidade do leite para os processamentos mais exigentes seria uma alternativa. Ver Portaria 392/2021 publicada pelo MAPA.

Em relação à análise quantitativa, reanálise de dados e meta-análise dos dados qualitativos selecionados pela revisão sistemática, vale ressaltar que há uma falta de padronização na metodologia dos trabalhos, mesmo quando o objetivo é semelhante, ou até mesmo igual, tanto na coleta quanto na apresentação dos dados, tal fato culmina na elaboração, ainda tímida, de trabalhos com esse perfil estatístico na área de qualidade do leite. Uma área que na verdade tem uma abundância de dados de amostras de animais individuais, tanto em nível de propriedade, quanto de indústria, que possuem um potencial para que mais trabalhos com esse perfil possam ser gerados, sabendo que a meta análise é uma ferramenta excelente para que se evite a replicação de objetivos e que se possa obter uma resposta consistente e convergente sobre tal assunto.

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ANEXOS

PRISMA – Checklist *From:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit: **www.prisma-statement.org**.

Page 2 of 2

Título- Identifique o relatório como uma revisão sistemática, meta-análise ou ambas

Abstract

Estrutura do sumário- Tem que fornecer: antecedentes; Objetivos; fontes de dados; estudar critérios de elegibilidade, participantes e intervenções; métodos de avaliação e síntese de estudos; resultados; limitações; conclusões e implicações das principais conclusões; número de registro da revisão sistemática.

Introdução

Fundamentação- Descreva a justificativa da revisão no contexto do que já é conhecido.

Objetivos-Forneça uma declaração explícita das perguntas que estão sendo tratadas com referência aos participantes, intervenções, comparações, resultados e desenho do estudo (PICOS).

Métodos

Protocolo e registro- Indique se existe um protocolo de revisão, se e onde pode ser acessado (por exemplo, endereço da Web) e, se disponível, forneça informações de registro, incluindo o número de registro.

Critérios de elegibilidade- Especifique as características do estudo (por exemplo, PICOS, duração do acompanhamento) e as características do relatório (por exemplo, anos considerados, idioma, status da publicação) usadas como critérios de elegibilidade, fornecendo justificativa.

Fontes de informação- Descreva todas as fontes de informação (por exemplo, bancos de dados com datas de cobertura, entre em contato com os autores do estudo para identificar estudos adicionais) na pesquisa e na data da última pesquisa.

Procura- Apresente uma estratégia de pesquisa eletrônica completa para pelo menos um banco de dados, incluindo os limites utilizados, para que possa ser repetido.

Seleção dos estudos- Declare o processo de seleção de estudos (ou seja, triagem, elegibilidade, incluídos na revisão sistemática e, se aplicável, incluídos na meta-análise).

Processo de coleta de dados-Descreva o método de extração de dados de relatórios (por exemplo, formulários pilotados, independentemente, em duplicado) e quaisquer processos para obter e confirmar dados dos investigadores.

Itens de dados- Liste e defina todas as variáveis para as quais os dados foram buscados (por exemplo, PICOS, fontes de financiamento) e quaisquer suposições e simplificações feitas.

Risco de viés em estudos individuais- Descreva os métodos usados para avaliar o risco de viés de estudos individuais (incluindo a especificação de se isso foi feito no nível do estudo ou do resultado) e como essas informações devem ser usadas em qualquer síntese de dados.

Medidas de resumo- Indique as principais medidas resumidas (por exemplo, razão de risco, diferença de médias).

Síntese dos resultados- Descreva os métodos de manipulação de dados e combinação de resultados de estudos, se realizados, incluindo medidas de consistência (por exemplo, I2) para cada meta-análise.

Risco de viés entre os estudos- Especifique qualquer avaliação do risco de viés que possa afetar a evidência cumulativa (por exemplo, viés de publicação, relatório seletivo nos estudos).

Análises adicionais- Descreva métodos de análises adicionais (por exemplo, análises de sensibilidade ou subgrupo, meta-regressão), se realizadas, indicando quais foram pré-especificadas.

Resultados

Seleção dos estudos- Forneça um número de estudos selecionados, avaliados quanto à elegibilidade e incluídos na revisão, com motivos de exclusão em cada estágio, idealmente com um fluxograma.

Características do estudo- Para cada estudo, apresente características para as quais os dados foram extraídos (por exemplo, tamanho do estudo, PICOS, período de acompanhamento) e forneça as citações.

Risco de viés nos estudos- Apresentar dados sobre o risco de viés de cada estudo e, se disponível, qualquer avaliação do nível de resultado (ver item 12).

Resultados de estudos individuais- Para todos os resultados considerados (benefícios ou malefícios), apresente, para cada estudo: (a) dados resumidos simples para cada grupo de intervenção (b) estimativas de efeito e intervalos de confiança, idealmente com uma parcela da floresta.

Síntese dos resultados- Apresente os resultados de cada meta-análise realizada, incluindo intervalos de confiança e medidas de consistência.

Risco de viés entre os estudos- Apresente os resultados de qualquer avaliação do risco de viés entre os estudos (ver Item 15).

Análise adicional- Forneça resultados de análises adicionais, se realizadas (por exemplo, análises de sensibilidade ou subgrupo, meta-regressão [ver Item 16]).

Discussão

Resumo da evidência- Resuma as principais conclusões, incluindo a força das evidências para cada resultado principal; considere sua relevância para grupos-chave (por exemplo, provedores de serviços de saúde, usuários e formuladores de políticas).

Limitações- Discuta as limitações no nível de estudo e resultado (por exemplo, risco de viés) e no nível da revisão (por exemplo, recuperação incompleta da pesquisa identificada, viés de relatório).

Conclusões- Forneça uma interpretação geral dos resultados no contexto de outras evidências e implicações para pesquisas futuras.

Financiamento

Financiamento- Descrever fontes de financiamento para a revisão sistemática e outro suporte (por exemplo, fornecimento de dados); papel dos financiadores para a revisão sistemática.

APÊNDICES

Normas utilizadas para a preparação dos capítulos I, III e IV

Guidance for preparing manuscripts for submission to JDR (Journal Dairy Research)

Manuscript types

Manuscript lengths are quoted in text equivalents (TEQ) where one word is one TEQ, one figure is 250 TEQ and one table is 250 TEQ. We encourage conciseness, and strongly recommend the use of a Supplementary File for both Research Papers and Research Communications.

Research Papers

Max length: around 6000 TEQ

Optimum length: as short as possible

Hypothesis driven: yes

Descriptive: not normally

Impact: international

Citations: minimal essential

Peer review: 6 week target

First view online: yes

Publication: first available issue

Research Communications

Max length: around 2500 TEQ

Optimum length: 2000 TEQ or less

Hypothesis driven: yes

Descriptive: yes

Impact: international or national

Citations: around 10 maximum

Peer review: 6 weeks target

First view online: yes

Publication: first available issue

Supplementary Files

JDR strongly encourages brevity and clarity. To keep your manuscript as short as possible, please make full use of our Supplementary File option.

- Figures and tables included with the main manuscript must be essential for the understanding of the research.
- The materials and methods section of the main manuscript must clearly state how the research was done, but need not give full details of established methods or analyses.
- Detailed methodologies (to allow repetition) and data files (figures, tables) that report supporting or purely descriptive information should be supplied as a separate supplementary file

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To ensure that we can publish as much high quality research as possible, we have a strict policy of encouraging shorter, concise articles that report novel findings which are highly relevant to our scope and are likely to have significant international or national impact. We have two formats: **Full Papers** report hypothesis led research, in other words, a hypothesis must be presented and tested and the conclusions must clearly indicate whether or not the hypothesis was supported by the experimental results. **Research Communications** are of equal scientific merit and report high-quality hypothesis-led or descriptive research in a way that focuses attention on the novel findings. This is our preferred category for most research. For both types of article we strongly encourage the use of a Supplementary File to provide supporting information such as non-essential data and detailed methodologies. This file can be of any length and is published online linked to the main article. Providing a Supplementary File ensures that the main article is as concise as possible.

Layout of Research Paper manuscripts

The manuscript should generally be divided as follows:

• Cover sheet should give the title of the article, names of the authors each with one forename together with their affiliations, a shortened version of the title suitable as a heading, and the name and email of the author to whom correspondence and proofs should be sent.

• Abstract, preferably not more than 300 words, should encapsulate the whole paper, showing clearly the new knowledge acquired. The first line of the summary should identify the article as a Research Paper and present the objectives, preferably in the form of a hypothesis (eg This Research Paper addresses the hypothesis that…). Without using separate sections, the Abstract should briefly explain what was done, why it was done, how it was done and what was found. Results and conclusions should be clearly stated, but the Abstract should not contain individual data values unless this is essential to the conclusions.

• Keywords: up to 5 keywords must be supplied

• Introduction should not have a heading. It should not contain a full review of the literature, but should help the non-specialist to understand why the subject of enquiry is interesting or important, why the authors have chosen the approach described and what the likely impact of the research will be. The objectives must be clearly stated, preferably in the form of a hypothesis.

• Materials and Methods section should be sufficiently informative to allow the reader to understand what was done, but should not contain the detail needed to allow repetition (this should be given in the Supplementary File). Proper reference must be made to the Supplementary Materials and Methods.

• Results should be as concise as possible, without repetition or inclusion of irrelevant material. Tables and illustrations should be used efficiently. All data reported must directly relate to the understanding of the research objectives and conclusions. Supporting or confirmatory data that corroborate the conclusions without being essential to them should be presented separately as part of the Supplementary File. Proper reference must be made to this Supplementary Data.

• Discussion should not repeat the results but discuss their significance. Refer to existing or accepted knowledge in the present tense and the authors' work in the past tense; the difference in tense should clearly show the authors' contribution. A separate conclusion is not necessary but authors should summarize their main conclusions briefly at the end of the Discussion. A combined Results and Discussion is acceptable but not preferred.

• Acknowledgements of financial support, technical assistance and so on are given in a separate paragraph. It is the responsibility of the authors to ensure that individuals or organizations acknowledged as providing materials or otherwise are willing to be identified.

• References must be consistent and must use the style described below.

• Tables and table legends, following the style described below.

• Figure legends sufficient to allow the figure to be understood without reference to the text

• Figures should be produced using an editable software and copied into the Word document. Please remember that the complete manuscript should be submitted as a single document.

References

Refer to a recent issue and ensure that your reference citations comply with Journal style. References should be given in the text as Brown & Jones (1987) or (Schmidt, 1985; Nakamura et al.1989); the first author with et al. is used for papers with three or more authors. Where necessary, papers are distinguished as Lenoir (1988a), (Litov et al. 1990a, b). When several references appear together in the text, cite them in chronological order, and alphabetically within years. The Reference list at the end of the paper, which should begin on a fresh page, is given in strict alphabetical order and uses the minimum of punctuation. Each reference should contain authors' names, with initials (in capitals), the year, the title of the paper, the name of the journal in full, the volume and the page range. Titles of articles originally published in another language should be given in English translation, and this indicated by the use of square brackets. References to books should include the town of publication and the publisher, with editor(s) and volume and edition number where appropriate. Unpublished work should be given in the text (use authors' initials and surname) and not in the Reference list. You are reminded that it is your responsibility to check all references.

Data presentation

Choose the most economical form of data presentation, remembering that this could include data presented briefly in the text. All data included in the manuscript must directly relate to the hypothesis or objectives and be essential to establish the conclusions. Confirmatory or supportive data that corroborate the conclusions should not be given in the manuscript, but as part of the Supplementary File. For investigative research, avoid including in the main text large tables and figures that are comprised mainly of data that do not differ significantly between treatments. For descriptive research, use the Supplementary File for all apart from the most important observations.

Tables

Tables should be numbered and carry headings enabling them to be understood without reference to the main text. Any abbreviations should be defined. Each Table should be typed separately at the end of the main text, but their approximate position should be indicated by a marginal mark (eg Table 1 near here). Symbols for footnotes should avoid use of *, **, etc, which should be used to indicate levels of significance. Additional Tables given in the Supplementary File should follow the same conventions and should be numbered Supplementary Table S1, S2 etc. Ensure that the manuscript includes proper reference to each Supplementary Table.

Figures and Illustrations

Figures should be numbered and the combination of figure and legend should be comprehensible without reference to the main text. Figures must be prepared using an editable file format and then copied into the Word document. Data points should be indicated by clearly distinguishable symbols. Illustrations such as photographic images should be accompanied by a legend as above, with scale bars if appropriate. Additional Figures given in the Supplementary File should follow the same conventions and should be numbered Supplementary Figure S1, S2 etc. Ensure that the manuscript includes proper reference to each Supplementary Figure. Colour figures and artwork submitted to the Journal as part of the manuscript will be published online free of charge. If you request colour figures in the printed version, you will be contacted by CCC-Rightslink who are acting on our behalf to collect Author Charges. Please follow their instructions in order to avoid any delay in the publication of your article.

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Authors should, where possible, discuss their work with a statistician at an early stage and give attention to sample size. Individual results should not normally be given. The methods of statistical analysis should be clearly described; a suitable reference is adequate. Authors should make it clear whether they are quoting SED, SEM, SD, SE and so on. Any statement that two groups of values are different should be supported by the level of significance involved. Differences should not be claimed or implied if $P > 0.05$.

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Authors should consult a current issue for guidance. Useful information on biochemical nomenclature and permitted acronyms can be found in Biochemical Journal 169, 11-14 and on nutrient nomenclature in the British Journal of Nutrition. If authors use other abbreviations or acronyms, they should be defined at first mention, and their number restricted to ensure that the text is readable. Always use Arabic numerals with units; otherwise use words for 1-10 and figures for more than 10, (e.g. 3 weeks, three cows, 34 sheep) but avoid mixed lists. Time should be given by the 24 h clock, e.g. 14.15, without h or hours.

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VITA

Lisiane da Silveira Garcia é brasileira, nascida em 9 de junho de 1995, em Porto Alegre/RS, filha de Carla Beatriz da Silveira Garcia e Luciano Weisheimer Garcia. Realizou seus estudos de educação infantil, fundamental e médio no Instituto de Educação Júlio César Ribeiro de Souza, no município de Alvorada. Em 2014 ingressou no curso de Agronomia na Universidade Federal do Rio Grande do Sul, se formando no ano de 2019. Em abril de 2020 iniciou o Mestrado Acadêmico no Programa de Pós Graduação em Zootecnia vinculado a Universidade Federal do Rio Grande do Sul, sob orientação da Professora Doutora Vivian Fischer, sendo a área de concentração de Nutrição de Ruminantes, na linha de pesquisa de Qualidade do Leite.