UNIVERSIDADE DO VALE DO TAQUARI- UNIVATES
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DOUTORADO EM AMBIENTE E DESENVOLVIMENTO

QUALIDADE DO LEITE BOVINO DAS PROPRIEDADES PRODUTORAS DE LEITE E DAS INDÚSTRIAS DO VALE DO TAQUARI-RS

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Lajeado/RS, janeiro de 2023

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Tese apresentada ao Programa de Pós-graduação em Ambiente e Desenvolvimento (PPGAD), da Universidade do Vale do Taquari - Univates, como parte da exigência para obtenção do grau de Doutora em Ciências: Ambiente e Desenvolvimento, na Área de concentração Espaço, Ambiente e Sociedade, e na linha de pesquisa Ecologia.

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RESUMO

A região do Vale do Taquari é uma das principais produtoras de leite do Rio Grande do Sul. A qualidade do leite produzido pode ser influenciada por diversos fatores associados ao manejo, obtenção, transporte ou armazenamento do leite. A presente tese teve como objetivo avaliar a qualidade do leite cru refrigerado coletado nas propriedades produtoras de leite dos municípios do Vale do Taquari – RS, e dos leites cru refrigerado, pasteurizado e esterilizado pelo processo *Ultra Hight Temperature* (UHT) de indústrias dessa mesma região. Foram realizadas análises microbiológicas: contagem bacteriana total (CBT), contagem de mesófilos, contagem de psicrotróficos e análises de coliformes totais e termotolerantes. Análises físico-químicas e de composição do leite: acidez, densidade relativa, temperatura, proteína, gordura, lactose, extrato seco total (EST) ou sólidos totais (ST) e extrato seco desengordurado (ESD) ou sólidos não-gordurosos (SNG), para os três tipos de leite. Adicionalmente, no leite cru refrigerado das propriedades e dos caminhões tanque das indústrias realizou-se a contagem de células somáticas (CCS) e o teste de álcool alizarol. Foi realizada também a análise do microbiota, por meio do sequenciamento de alto rendimento do gene ribossomal 16S para os leites cru refrigerado, pasteurizado e esterilizado das indústrias. Os dados obtidos nas análises foram comparados com o que preceitua a legislação vigente, Instrução Normativa nº 76 e Instrução Normativa n° 77 e com a Portaria n° 370, do Ministério da Agricultura, Pecuária e Abastecimento (MAPA). As coletas das amostras ocorreram em 33 propriedades produtoras de leite e duas indústrias beneficiadoras da região. Os resultados mostraram que há uma grande diversidade de gêneros e espécies, sendo que a indústria 1 demonstrou maiores quantidades de microrganismos. Os resultados das análises físico-químicas demonstraram que apenas duas propriedades estavam com a acidez acima do limite estabelecido pela legislação vigente. A indústria 1 apresentou acidez acima do limite nos três tipos de leite, alizarol positivo no leite cru refrigerado e densidade fora do estabelecido para o leite pasteurizado. Os parâmetros de composição do leite demostraram que mais da metade das propriedades (53%) e as duas indústrias estavam com a CCS acima do estabelecido. As análises microbiológicas demostraram CBT acima do permitido na indústria 1 e em nove das 33 propriedades analisadas. A quantidade de microrganismos psicrotróficos foi maior nas indústrias em comparação com as propriedades e ficou acima do estabelecido por autores da área. O leite cru refrigerado das indústrias apresentou maiores quantidades de CBT, psicrotróficos e coliformes totais e termotolerantes do que o leite cru refrigerado das propriedades produtoras de leite. A indústria 1 apresentou maiores quantidade de CCS, CBT, mesófilos e psicrotróficos que a indústria 2. Os parâmetros físico-químicos e microbiológicos são extremamente importantes para comprovar a qualidade do leite produzido e rastrear possíveis falhas no processamento. A utilização de ferramentas moleculares, como o sequenciamento de alto rendimento, torna o diagnóstico da qualidade do leite mais eficiente e minucioso, podendo ainda ser utilizado para a melhoria do processo produtivo.

Palavras-chave: Coliformes. Mesófilos. Psicrotróficos. Análises físico-químicas. Microbiota do leite. Composição do leite.

ABSTRACT

The Vale do Taquari region is one of the main milk producers in Rio Grande do Sul. The quality of the milk produced can be influenced by several factors associated with handling, obtaining, transporting or storing the milk. This thesis aimed to evaluate the quality of refrigerated raw milk collected in milk producing properties in the municipalities of Vale do Taquari - RS, and of refrigerated, pasteurized and sterilized raw milk by the Ultra High Temperature (UHT) process from industries in the same region.Microbiological analyzes were carried out: total bacterial count (TBC), mesophilic count, psychrotrophic count and analysis of total and thermotolerant coliforms. Physical-chemical and composition analysis of milk: acidity, relative density, temperature, protein, fat, lactose, total dry extract (EST) or total solids (ST) and defatted dry extract (ESD) or non-fat solids (SNG), for the three types of milk. Additionally, the somatic cell count (SCC) and the alizarol alcohol test were performed on refrigerated raw milk from farms and from tank trucks. Microbiota analysis was also carried out, through high-throughput sequencing of the 16S ribosomal gene for refrigerated, pasteurized and sterilized raw milk from industries. The data obtained in the analyzes were compared with the provisions of current legislation, Normative Instruction No. 76 and Normative Instruction No. 77 and with Ordinance No. 370, of the Ministry of Agriculture, Livestock and Supply (MAPA). Samples were collected from 33 milk producing properties and two processing industries in the region. The results showed that there is a great diversity of genera and species, and industry 1 showed greater amounts of microorganisms. The results of the physical-chemical analyzes showed that only two properties had acidity above the limit established by current legislation. Industry 1 had acidity above the limit in the three types of milk, positive alizarol in refrigerated raw milk and density outside the established range for pasteurized milk. The milk composition parameters showed that more than half of the properties (53%) and the two industries had the CCS above the established. Microbiological analyzes showed CBT above the permitted level in industry 1 and in nine of the 33 properties analyzed. The amount of psychrotrophic microorganisms was higher in the industries compared to the properties and was above the established by authors in the area. Raw refrigerated milk from industries showed higher amounts of CBT, psychrotrophs and total and thermotolerant coliforms than raw refrigerated milk from dairy farms. Industry 1 had higher amounts of CCS, CBT, mesophiles and psychrotrophs than industry 2. The physical-chemical and microbiological parameters are extremely important to prove the quality of the milk produced and to track possible failures in processing. The use of molecular tools, such as high-throughput sequencing, makes the diagnosis of milk quality more efficient and thorough, and can also be used to improve the production process.

Key- words: Coliforms. Mesophiles. Psychrotrophs. Physicochemical analysis; Microbiota; Milk composition.

SUMÁRIO

1. APRESENTAÇÃO

O leite é um alimento rico, que possui características nutricionais imprescindíveis ao ser humano. Pelo fato de ser um alimento essencial, o leite também é um dos principais produtos agropecuários (CARVALHO *et al.*, 2022). Segundo a *Food and Agriculture Organization of the United Nations* (FAO, 2021), a produção de leite é uma ferramenta importante para a economia e para o desenvolvimento dos países. Além disso, é imprescindível ao desenvolvimento social, pois em todo o mundo, cerca de 150 milhões de famílias trabalham na produção de leite. Essa produção é caracterizada, em sua maioria, por pequenos produtores que vivem em países em desenvolvimento, sendo esta a principal atividade para a subsistência.

O Brasil é considerado o quinto maior produtor de leite do mundo e possui a agropecuária leiteira como uma de suas principais atividades econômicas (FAO, 2021). Durante o ano de 2021, a produção de leite cru refrigerado no Brasil foi de 25,3 bilhões de litros (IBGE, 2021) e no segundo trimestre de 2022, a produção chegou a 5,38 bilhões de litros (IBGE, 2022).

O estado do Rio Grande do Sul (RS), é considerado o segundo maior produtor do país, responsável por aproximadamente 885 milhões de litros (IBGE, 2022). Dentre as regiões do estado do RS, o Vale do Taquari é responsável por grande parte da produção estadual de leite. Esta região do Vale do Taquari produz, em média, 4.406.428 litros por ano e mais de um milhão de litros de leite por dia, sendo a terceira maior bacia leiteira do estado (SPGG, 2020).

A produção de leite no Vale do Taquari, tem grande importância econômica e social, pois permite a obtenção de renda às famílias que dependem de agricultura familiar, além de ser responsável pela manutenção da população no campo. O leite produzido nas propriedades produtoras de leite do VT é vendido para indústrias, também regionais, que são responsáveis pelo seu beneficiamento e distribuição.

A compreensão detalhada sobre as mais variadas etapas de produção do leite, desde a propriedade produtora de leite, passando pelo transporte nos caminhões-tanque e, posteriormente, pelos processos de beneficiamento dentro da indústria, torna-se muito importante para identificar eventuais falhas e buscar melhorias em toda a cadeia produtiva. Isso evita também situações de desperdício de alimentos, nos casos em que o leite, por estar com sua qualidade comprometida, precise ser descartado. Além disso, um leite de boa qualidade é imprescindível para a saúde do consumidor e uma qualidade prejudicada, interfere, além da saúde, no retorno econômico de toda a cadeia produtiva.

A qualidade do leite, quando prejudicada, pode causar contaminação aos consumidores, provocando até mesmo surtos de Doenças de Transmissão Hídrica e Alimentar (DTHA), sendo a doença diarreica a mais comum dentre as DTHAs. Há uma estimativa de que uma em cada 10 pessoas adoece devido ao consumo de alimento contaminado. Os contaminantes podem ter diversas origens: biológica (bactérias, vírus, parasitas e príons), química (metais pesados) e física (fragmentos de diferentes materiais) (HEMALATA; VIRUPAKSHAIAH, 2016).

Para que o leite cru refrigerado possa ser consumido, ocorrem na indústria, processos de beneficiamento. Esses processos produzem tipos diferentes de leite e melhoram a qualidade do mesmo. O tipo de leite produzido depende do processo de aquecimento aplicado: pasteurização ou esterilização por *Ultra Hight Temperature* (UHT). Esses processos de aquecimento eliminam patógenos e aumentam a vida útil do produto, quando mantido em embalagens fechadas (BRASIL, 2018b).

Ao longo dos anos, diversas normativas do Ministério da Agricultura, Pecuária e Abastecimento (MAPA), foram sendo desenvolvidas e sancionadas, para que a qualidade do leite pudesse ser melhor interpretada e implementada. A primeira delas foi a Portaria DILEI/SIPA/SNAD/MA nº 08, de 26 de junho de 1984 (BRASIL, 1984). A Instrução Normativa (IN) nº 51, de 18 de setembro de 2002 (BRASIL, 2002), foi um marco que determinou limites dos parâmetros do leite. Após essa, surgiram outras normativas que foram evoluindo ao longo dos anos e aprimorando cada vez mais a avaliação da qualidade do leite bovino no Brasil. Dentre elas, pode-se citar a IN n°62, de 29 de dezembro de 2011 (BRASIL, 2011), que determinou, além dos parâmetros para avaliação do leite, as metodologias que deveriam ser utilizadas para essa avaliação.

Atualmente, os parâmetros de qualidade do leite, bem como seus limites, são determinados pela IN n° 76/2018 (BRASIL, 2018a) e pela IN n° 77/2018 (BRASIL, 2018b), ambas para o leite cru refrigerado de propriedades e indústrias e para o leite pasteurizado, e pela Portaria n° 370/1997 (BRASIL, 1997), para o leite UHT.

Os parâmetros utilizados para avaliar a qualidade do leite são obtidos por meio de análises da composição: contagem de células somáticas (CCS), lactose, proteína, gordura, extrato seco total (EST) ou sólidos totais (ST) e extrato seco desengordurado (ESD) ou sólidos não-gordurosos (SNG), análises físico-químicas: alizarol, temperatura, acidez e densidade, e análises microbiológicas: contagem bacteriana total (CBT), realizada apenas no leite cru refrigerado de propriedades produtoras de leite e indústrias. A análise desses parâmetros é realizada pela Rede Brasileira de Qualidade do Leite (RBQL) (BRASIL, 2018b), composta por uma rede de laboratórios credenciados.

Aos aspectos descritos acima, pode-se adicionar a contagem de microrganismos mesófilos, a contagem de microrganismos psicrotróficos e as análises de coliformes totais e termotolerantes para a verificação da qualidade do leite produzido. Além do sequenciamento de alto rendimento, que possibilita a verificação da microbiota do leite e tem sido uma ferramenta importante para a análise mais específica e minuciosa do leite produzido.

A tese aqui apresentada está inserida dentro da linha de pesquisa do Programa de Pós-graduação em Ambiente e Desenvolvimento (PPGAD), Ecologia. Esse trabalho foi desenvolvido dentro das atividades de uma pesquisa da instituição, que estuda as propriedades produtoras de leite do Vale do Taquari- RS, intitulada “Sustentabilidade em Propriedades Produtoras de Leite”.

O tema da presente tese é a qualidade do leite cru refrigerado das propriedades produtoras de leite, do leite transportado e do leite beneficiado nas indústrias do Vale do Taquari - RS. O problema de pesquisa foi: Qual a qualidade do leite cru refrigerado das propriedades produtoras de leite, do leite transportado e do leite beneficiado nas indústrias do Vale do Taquari - RS? A tese inicial foi de que a qualidade microbiológica e físico-química e a composição do leite da região do Vale do Taquari atendem ao esperado pela legislação, mas podem ser melhoradas por meio da avaliação de parâmetros não determinados pela legislação e pela análise da sua microbiota. O objetivo geral foi avaliar e comparar a qualidade do leite cru das propriedades produtoras de leite, do leite transportado e do leite beneficiado nas indústrias no Vale

do Taquari - RS. Para tal, os objetivos específicos desta pesquisa foram:

- Sintetizar, por meio de uma revisão integrativa, os principais dados existentes a respeito da qualidade físico-química e microbiológica do leite bovino no Brasil;
- Avaliar a qualidade do leite cru refrigerado das propriedades produtoras de leite e das indústrias do Vale do Taquari – RS, por meio de análises microbiológicas e de composição do leite: contagem bacteriana total, contagem de microrganismos psicrotróficos, análise de coliformes totais e termotolerantes e contagem de células somáticas;
- Avaliar a qualidade do leite pasteurizado e do leite esterilizado das indústrias do Vale do Taquari – RS, por meio de contagem de microrganismos mesófilos, contagem de microrganismos psicrotróficos e análise de coliformes totais e termotolerantes;
- Avaliar a qualidade do leite cru refrigerado das propriedades produtoras de leite e dos leites cru refrigerado, pasteurizado e esterilizado das indústrias do Vale do Taquari – RS, por meio de análises de composição do leite: lactose, proteínas, gorduras, sólidos não-gordurosos e sólidos totais;
- Avaliar a qualidade do leite cru refrigerado das propriedades produtoras de leite e dos leites cru refrigerado, pasteurizado e esterilizado das indústrias do Vale do Taquari – RS, por meio de análises físico-químicas: acidez, alizarol, densidade relativa e temperatura;
- Identificar os microrganismos presentes na microbiota do leite cru refrigerado, pasteurizado e esterilizado das indústrias do Vale do Taquari -RS, por meio de sequenciamento de alto rendimento;
- Comparar a qualidade do leite cru refrigerado e dos leites processados das indústrias do Vale do Taquari – RS.

As coletas ocorreram em 33 propriedades produtoras de leite, sendo uma propriedade de cada município (33 municípios) pertencente ao Vale do Taquari (VT) e em duas indústrias beneficiadoras envolvidas no recolhimento e beneficiamento do leite da região. As propriedades faziam parte do projeto “Sustentabilidade em Propriedades Produtoras de Leite”, que possui um total de 104 propriedades cadastradas. Como critérios de inclusão das propriedades foram observados aspectos como a aceitação da coleta por parte dos produtores e a localização da propriedade, para facilitar o acesso, já que eram coletadas três propriedades (em três municípios) em cada vez. Os produtores possuem propriedades diversificadas, conciliando a

produção de leite com outras atividades agrícolas e agropecuárias. O volume de produção das propriedades é bem variado, de 160 litros/dia a 6.500 litros/dia, porém em sua maioria (26 propriedades) a produção diária não ultrapassa 1.000 litros/dia. Em relação às indústrias, realizou-se um contato telefônico e por e-mail com todas as indústrias beneficiadoras do VT, sendo que duas permitiram a coleta das amostras.

Foram realizadas análises microbiológicas de coliformes totais e termotolerantes, contagem bacteriana total (CBT), contagem de microrganismos mesófilos e psicrotróficos, análises de composição do leite: contagem de células somáticas (CCS), lactose, proteína, gordura, sólidos totais, sólidos não gordurosos, análises físico-químicas: acidez, alizarol, densidade relativa e temperatura e da microbiota do leite das indústrias. Realizou-se um comparativo da qualidade dos três tipos de leite coletado, para avaliar a eficiência do transporte e processamento. Os dados obtidos nas análises foram comparados com o que preceitua a legislação vigente, IN nº 76/2018 (BRASIL, 2018a) e Portaria n° 370/1997 (BRASIL, 1997).

1.1 Estrutura da Tese

Esta Tese foi estruturada sob o formato de artigos científicos, conforme dispõe a Resolução no Resolução 070/Consun/Univates (Regimento do Programa de Pós-graduação em Ambiente e Desenvolvimento - PPGAD), de 31 de agosto de 2018, em seu artigo 30, inciso III, que versa sobre o formato alternativo do trabalho de conclusão de curso do PPGAD, da Universidade do Vale do Taquari - Univates.

No caso da presente pesquisa doutoral, utilizou-se o formato de artigos verticais ou sequenciais. Foram produzidos cinco artigos, sendo que o primeiro artigo: “Quality of bovine milk produced in Brazil – Physical-chemical and microbiological parameters: an integrative review” contempla o primeiro objetivo específico desta pesquisa. Os outros quatro artigos produzidos (artigos 2, 3, 4 e 5) contemplam os objetivos específicos envolvidos na pesquisa de campo e foram elaborados com os dados produzidos a partir das coletas das amostras de leite das propriedades produtoras de leite e das indústrias beneficiadoras da região do Vale do Taquari (Quadro 1). Além dos cinco artigos, foi também produzido um capítulo de livro, “A qualidade do leite bovino no Brasil: diretrizes e mudanças”, publicado no Livro do Programa de Pós-graduação em Sistemas Ambientais Sustentáveis (PPGSAS).

Quadro 1 – Estrutura da tese com os respectivos artigos produzidos por meio da coleta e análise de dados

Objetivos Específicos	Artigos	Principais Resultados
1. Sintetizar, por meio de uma revisão integrativa, os principais dados existentes a respeito da qualidade físico-química e microbiológica do leite bovino no Brasil; 2. Avaliar a qualidade do leite cru refrigerado das propriedades produtoras de leite e das indústrias do Vale do Taquari – RS, por meio de análises microbiológicas e de composição do leite: contagem bacteriana total, contagem de microrganismos psicrotróficos, análise de coliformes totais e termotolerantes e contagem de células somáticas; 3. Avaliar a qualidade do leite pasteurizado e do leite esterilizado das indústrias do Vale do Taquari – RS, por meio de contagem de microrganismos mesófilos, contagem de microrganismos psicrotróficos e análise de coliformes totais e termotolerantes; 4. Avaliar a qualidade do leite cru refrigerado das propriedades produtoras de leite e dos leites cru refrigerado, pasteurizado e esterilizado das indústrias do Vale do Taquari – RS, por meio de análises de composição do leite: lactose, proteínas, gorduras, sólidos não-gordurosos, sólidos totais; 5. Avaliar a qualidade do leite cru refrigerado das propriedades produtoras de leite e dos leites cru refrigerado, pasteurizado e esterilizado das indústrias do Vale do Taquari – RS, por meio de análises físico-químicas: acidez, alizarol, densidade relativa e temperatura; 6. Identificar os	Artigo 1: “Quality of bovine milk produced in Brazil – Physical-chemical and microbiological parameters: an integrative review”, aborda o objetivo 1.	-Os aspectos físico-químicos do leite não demonstraram alterações significativas na maior parte das amostras analisadas; - A análise de conteúdo dos artigos demonstrou que 93% deles relataram alterações microbiológicas no leite, evidenciando a necessidade de adoção de boas práticas agropecuárias e de fabricação, além de formas eficazes de armazenamento do leite coletado.
	Artigo 2: “Physicochemical and microbiological quality of bovine milk from Vale do Taquari in Rio Grande do Sul”, aborda os objetivos 2, 3, 4, 5 e 7	-Das 33 propriedades, duas apresentaram leite com acidez e três apresentaram leite CBT acima do estabelecido; -O leite da indústria 1 apresentou acidez, CBT e densidade fora dos padrões estabelecidos; -As duas indústrias e 53,2% das propriedades CCS acima do determinado pela legislação; -O leite das indústrias demonstrou maiores quantidades de CCS, CBT, psicrotróficos e coliformes totais e termotolerantes que o leite das propriedades produtoras de leite e o leite da indústria 1 apresentou maiores quantidades que a indústria 2, nos parâmetros microbiológicos.
	Artigo 3: “Quality of refrigerated, pasteurized and sterilized raw bovine milk from industries in Vale do Taquari – RS”, aborda os objetivos 2, 3, 4, 5, 6 e 7.	-Ambas as indústrias apresentaram CCS acima do limite estabelecido para o leite cru refrigerado e níveis de psicrotróficos superior ao de mesófilos; -A indústria 1 apresentou acidez acima do limite nos três tipos de leite, CBT e densidade para o leite cru refrigerado e para o leite pasteurizado; -As amostras apresentaram ampla diversidade de gêneros, composta por psicrotolerantes, formadores de biofilmes, mastitogênicos e ácido láticos, além de gêneros considerados nocivos.
	Artigo 4: “Microbiological profile of raw refrigerated and processed bovine milk at dairy industries from Vale do Taquari – RS”, aborda os objetivos 6 e 7.	-O leite processado mostrou a presença de microrganismos benéficos como *Streptococcus thermophilus e Streptococcus macedonicus*. - O leite esterilizado mostrou a presença de microrganismos considerados nocivos como *Bacillus cereus group*, *Aeromonas dhakensis, Enterobacter bacterium* e *Acinetobacter haemolyticus.*
	Artigo 5: “Milk	-O leite cru refrigerado possui a maior

microrganismos presentes na microbiota do leite cru refrigerado, pasteurizado e esterilizado das indústrias do Vale do Taquari -RS, por meio de sequenciamento de alto rendimento; 7. Comparar a qualidade do leite cru refrigerado e dos leites processados das indústrias do Vale do Taquari – RS.	microbiota from dairy factories in the central region of Rio Grande do Sul, Brazil", aborda os objetivos 6 e 7.	quantidade de microrganismos nos dois laticínios, seguido do leite pasteurizado e pelo leite esterilizado pelo processo UHT, sucessivamente. O processamento do laticínio 2 mostrou-se mais eficiente, principalmente para o leite UHT, reduzindo consideravelmente a microbiota; -As amostras mostraram considerável diversidade microbiológica. Bactérias ácido-láticas como *Streptococcus macedonicus* foram encontradas no leite cru refrigerado e no leite pasteurizado e *Streptococcus termophilus*, no leite esterilizado; -Espécies nocivas como *Bacillus cereus group*, *Aeromonas dhakensis* e *Acinetobacter haemolyticus* foram encontrados no leite UHT de ambos os laticínios.

Elaborado pela autora.

No capítulo "Desenvolvimento" estão elencadas as produções científicas produzidas na presente tese, conforme mencionado acima, e apresentam o conjunto de resultados necessários a atender o objetivo principal do estudo. Os referidos artigos foram publicados ou submetidos em periódicos, de acordo com as determinações do PPGAD.

O capítulo denominado "Discussão Geral" apresenta os principais resultados elencados em cada artigo, relacionando-os com os objetivos específicos, bem como ao escopo geral deste estudo. Por fim, na seção "Considerações finais", evidenciam-se as conclusões deste estudo. Além disso, são abordadas as limitações desta pesquisa e as sugestões e recomendações para trabalhos futuros.

2. DESENVOLVIMENTO

Nesta seção estão apresentados o capítulo do livro e os cinco artigos produzidos na presente tese. O capítulo do livro **“**A qualidade do leite bovino no Brasil: diretrizes e mudanças” publicado no livro Sistemas Ambientais Sustentáveis e o artigo 1, artigo de revisão “Quality of bovine milk produced in Brazil – Physical-chemical and microbiological parameters: an integrative review” publicado na Revista Vigilância Sanitária em Debate: Sociedade, Ciência & Tecnologia contemplam a fundamentação teórica desta pesquisa.

O artigo 2, “Physicochemical and microbiological quality of bovine milk from Vale do Taquari in Rio Grande do Sul”, publicado na Revista Ciência Animal Brasileira, aborda a qualidade bioquímica, físico-química e microbiológica e do leite produzido nas propriedades produtoras de leite do Vale do Taquari – RS e a qualidade do leite cru refrigerado que chega na indústria por meio dos caminhões-tanque e dos leites processados pela pasteurização e pelo UHT.

O artigo 3, “Quality of refrigerated, pasteurized and sterilized raw bovine milk from industries in Vale do Taquari – RS”, publicado na Revista *Internacional Journal of Development Research*, aborda os parâmetros físico-químicos, microbiológicos e de composição do leite cru refrigerado, pasteurizado e esterilizado das indústrias da região do VT, além da análise da microbiota à nível de gêneros, realizado por meio do sequenciamento de alto rendimento.

O artigo 4, “Microbiological profile of raw refrigerated and processed bovine milk at dairy industries from Vale do Taquari – RS”, publicado na Revista Arquivos do Instituto Biológico, apresenta a análise da microbiota do leite cru refrigerado, pasteurizado e esterilizado pelo processo UHT das indústrias de laticínios da região do Vale do Taquari, à nível de espécies, elencando as principais espécies nos

diferentes tipos de leite, realizando um comparativo na quantidade de sequências a fim de avaliar a qualidade do leite e do processamento realizado nas indústrias.

O artigo 5, “Milk microbiota from dairy dactories in the central region of Rio Grande Do Sul, Brazil”, em avaliação na Revista Ciência e Agrotecnologia, apresenta a análise da microbiota dos três tipos de leite coletados nas indústrias de laticínios do Vale do Taquari realizando um comparativo entre as indústrias e os principais microrganismos encontrados.

A QUALIDADE DO LEITE BOVINO NO BRASIL: DIRETRIZES E MUDANÇAS

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Resumo: O Brasil está entre os cinco maiores produtores mundiais de leite bovino. O leite é um alimento rico em água e nutrientes, amplamente consumido pela população e por isso, precisa ser um produto de qualidade. A qualidade do leite produzido sofre a influência de diversos fatores, desde as etapas de produção nas propriedades produtoras de leite, até o beneficiamento nas indústrias de laticínios. Os parâmetros de qualidade do leite produzido são estabelecidos pela legislação vigente, Instrução Normativa nº 76 e Instrução Normativa nº 77, ambas de 2018 do Ministério da Agricultura, Pecuária e Abastecimento. Ao longo dos anos, diversas mudanças foram realizadas nas diretrizes que regulamentam a produção do leite, com o intuito de melhorar a sua qualidade. Esse capítulo tem como objetivo trazer um panorama sobre a qualidade do leite bovino produzido no Brasil, elencando os parâmetros definidos pela legislação, que são utilizados para determinação de sua qualidade e fazer um apanhado das mudanças nas diretrizes do leite ao longo dos anos. A avaliação da qualidade do leite é importante para promover melhorias em toda a cadeia produtiva assegurando a saúde do consumidor final e o retorno financeiro dos produtores.

Palavras-chave: Legislação. Parâmetros físico-químicos. Parâmetros microbiológicos.

1 INTRODUÇÃO

O Brasil é o 5° maior produtor mundial de leite do mundo, sendo responsável por cerca de 7% do total produzido (FAO, 2020). O leite é um alimento rico em diversos nutrientes, muito consumido pela população, principalmente nos primeiros anos de vida e que pode trazer inúmeros benefícios à saúde (STOPPE *et al.*, 2021), por ser fonte de proteínas, lipídios, lactose, sais minerais e vitaminas (LANGERIJT *et al.,* 2021). A qualidade do leite produzido precisa estar de acordo com os critérios estabelecidos pela legislação vigente, a fim de garantir a saúde do consumidor. Além disso, um leite com qualidade prejudicada acarreta em prejuízos financeiros ao produtor.

O leite produzido nas propriedades produtoras de leite recebe o nome de leite cru e precisa ser mantido a uma temperatura menor que 5 °C para evitar a proliferação dos microrganismos (BRASIL, 2018). Ao chegar à indústria de laticínios, esse leite passa pelo beneficiamento, produzindo o leite pasteurizado ou o leite *Ultra Hight Temperature* (UHT) (MACHADO *et al.*, 2017). O leite pasteurizado pode ser definido como leite fluido submetido a um dos processos de pasteurização, envasado automaticamente e destinado a consumo humano direto (BRASIL, 2018). A pasteurização lenta é realizada com a temperatura

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de 62 a 65 °C por 30 minutos e é permitida apenas para laticínios de pequeno porte. Já a pasteurização rápida, é realizada com temperatura de 72 a 75 °C por 15 a 20 segundos. Os dois processos devem inativar a enzima fosfatase alcalina do leite, mas manter a enzima peroxidase ainda ativa, garantindo o atendimento aos parâmetros estabelecidos (STARIKOF *et al.*, 2016). O leite UHT é entendido como leite homogeneizado submetido, durante 2 a 4 segundos, a uma temperatura de 130 ºC (BRASIL, 1997).

A qualidade do leite pode ser influenciada por diversos fatores, desde a sua produção até o seu beneficiamento e cada uma das etapas passa por um controle rigoroso de qualidade, tendo a sua própria legislação. Ao longo dos anos várias legislações foram publicadas, revogadas e/ou aprimoradas com o intuito de melhorar a qualidade do leite produzido, por meio de diretrizes da produção, transporte e beneficiamento do leite bovino produzido no Brasil.

O leite cru refrigerado e o leite pasteurizado possuem como legislação vigente as Instruções Normativas n° 76/2018 e n° 77/2018, ambas do Ministério da Agricultura, Pecuária e Abastecimento (MAPA), publicadas em 26 de novembro de 2018 (BRASIL, 2018), que estabelecem a identidade e as características de qualidade que o leite bovino deve apresentar e os critérios e procedimentos para a produção, acondicionamento, conservação, transporte, seleção e recepção do leite cru em estabelecimentos, respectivamente. O leite UHT possui como legislação vigente a Portaria n° 370/1997, do MAPA, de 04 de setembro de 1997 (BRASIL, 1997), que regulamenta a sua identidade e a sua qualidade.

Esse capítulo é parte teórica da tese intitulada "Qualidade do leite de propriedades produtoras de leite – do transporte ao processo nas indústrias no Vale do Taquari - RS", da doutoranda Thais Müller, vinculada ao Programa de Pós-Graduação em Ambiente e Desenvolvimento (PPGAD- Univates), orientada pela professora Dra. Claudete Rempel e coorientada pela professora Dra. Mônica Jachetti Maciel, e foi escrito em colaboração com a acadêmica de Medicina, Laura Gaspary. O objetivo deste capítulo é trazer um panorama sobre a qualidade do leite bovino produzido no Brasil, elencando os parâmetros analisados e definidos pela legislação vigente, que são utilizados para determinação de sua qualidade e fazer um apanhado das mudanças nas diretrizes do leite ao longo dos anos.

2 DESENVOLVIMENTO

2.1 Legislação e parâmetros de qualidade do leite ao longo dos anos

Ao longo dos anos, diversas normativas foram desenvolvidas e sancionadas, trazendo as diretrizes para a produção, transporte e processamento do leite produzido. A IN n° 77/2018, estabelece os critérios e procedimentos para a produção, acondicionamento, conservação, transporte, seleção e recepção do leite cru em estabelecimentos registrados no serviço de inspeção oficial, e a IN n° 76/2018, estabelece os limites para cada um dos parâmetros utilizados para a avaliação da qualidade do leite. Essas duas normativas do MAPA (BRASIL, 2018) ao entrarem em vigor, revogaram as antigas legislações sobre a produção de leite no Brasil:

- Portaria DILEI/SIPA/SNAD/MA nº 08, de 26 de junho de 1984;
- Instrução Normativa nº 51, de 18 de setembro de 2002;
- Instrução Normativa SDA/MAPA nº 22, de 07 de julho de 2009;
- Instrução Normativa nº 62, de 29 de dezembro de 2011;

- Instrução Normativa nº 07, de 03 de maio de 2016;
- Instrução Normativa nº 31, de 29 de junho de 2018.

A Portaria nº 08/1984, de 26 de junho de 1984 foi a primeira normativa sobre a qualidade do leite sancionada e utilizada no Brasil. Em setembro de 2002, entrou em vigor a IN nº 51/2002, que estabeleceu os regulamentos técnicos de produção, identidade e qualidade do leite tipo A, do leite tipo B, do leite tipo C, do leite pasteurizado e do leite cru refrigerado e o regulamento técnico da coleta de leite cru refrigerado e seu transporte a granel. Nessa normativa, a temperatura de refrigeração do leite cru tipo B deveria ser igual ou inferior a 4 ºC, podendo ser mantido na propriedade rural por até 48h. Ao ser transportado para estabelecimento industrial, o leite não poderia estar em temperatura superior a 7 ºC (BRASIL, 2002).

A IN n° 51/2002 estabeleceu os procedimentos do estabelecimento beneficiador com a realização de análises físico-químicas e microbiológicas para a avaliação da qualidade do leite. Conforme a normativa, as seguintes análises: estabilidade ao alizarol, contagem padrão de placas (CPP), contagem de células somáticas (CCS), índice crioscópico, extrato seco desengordurado (ESD) ou sólidos não gordurosos (SNG), densidade relativa, acidez titulável, gordura, teste redutase, resíduos de antibióticos, além da pesquisa de indicadores de fraudes e adulterações deveriam ser realizadas periodicamente. Essa normativa estabeleceu ainda que a medição da temperatura do leite cru refrigerado deveria ser realizada diariamente na propriedade rural e também na sua entrega ao estabelecimento beneficiador.

A IN n° 51/2002, estabeleceu também os valores de referência para os parâmetros avaliados, para o leite cru refrigerado e para o leite pasteurizado tipo A: acidez titulável entre 0,14 e 0,18 (g de ácido lático/100mL), densidade relativa entre 1,028 e 1,034 (15/15 ºC g/mL), índice crioscópico menor ou igual a 0,530 ºH (ou -0,512 ºC), teor mínimo de gordura de 3,0 g/100 g e ESD mínimo de 8,4 g/100g. Quanto à contagem padrão de placas, esta não poderia ultrapassar o máximo de 5 x 10^5 UFC/mL, e a contagem de células somáticas deveria ser de no máximo 6 x 10^5 CS/mL. Para o leite pasteurizado deveriam ser realizados ainda testes enzimáticos, apresentando prova de fosfatase negativa e prova de peroxidase positiva (BRASIL, 2002).

A IN SDA nº 22/2009, do MAPA, de 07 de julho de 2009, alterou o anexo IV da IN n° 51/2002 e estabeleceu que após a ordenha, o leite deveria ser imediatamente transportado do local de produção ao tanque de refrigeração de leite, sendo proibido o recebimento de leite previamente refrigerado. O tanque poderia ser comunitário e o titular do tanque precisaria ser inscrito no Cadastro Nacional de Produtores do Sistema de Informações Gerenciais do Serviço de Inspeção Federal (SIGSIF). Ao receber o leite no tanque de refrigeração deveria ser realizado o teste de alizarol e caso o leite estivesse positivo, indicando acidificação do mesmo, este não poderia ser adicionado ao tanque.

Em dezembro de 2011, a IN nº 62/2011, do MAPA, (BRASIL, 2011) entrou em vigor, estabelecendo novos regulamentos técnicos de produção, identidade e qualidade do leite. Essa normativa determinou que a granja leiteira deveria possuir, obrigatoriamente, equipamento para a ordenha mecânica, pré-filtragem e bombeamento até o tanque de depósito (localizado na dependência de beneficiamento e envase) em circuito fechado. Em 2016, a IN nº 07/2016 (BRASIL, 2016) alterou o Anexo II da IN n° 62/2011, estabelecendo que a temperatura do leite cru refrigerado não poderia exceder 7 ºC, na propriedade rural/tanque comunitário e 10 ºC, no estabelecimento processador.

A IN n° 62/2011 estabeleceu ainda que o Leite Tipo A é aquele com teor de gordura integral, semidesnatado ou desnatado, produzido, beneficiado e envasado na granja leiteira. Conforme a IN n° 62/2011, o leite pasteurizado do tipo A é: Integral, quando o teor de gordura mínimo for de 3,0 g/100 g, semidesnatado, quando o teor de gordura estiver entre 0,6 g/100 g e 2,9 g/100 g e desnatado quando possui o teor máximo de gordura for de 0,5 g/100 g. Para o processo de pasteurização, o leite deve sofrer aquecimento de 72 ºC a 75 ºC por 15s a 20s, seguido de resfriamento imediato até 4 ºC (BRASIL, 2011).

O controle da qualidade do leite deveria ser feito pelos procedimentos já determinados na IN n° 51/2002, tornando obrigatório analisar diariamente o leite cru refrigerado dos caminhões-tanques quanto a critérios como ESD, CPP, CCS, teores de gordura, acidez titulável, densidade relativa, índice crioscópico, alizarol, temperatura e pesquisa de resíduos de antibióticos (BRASIL, 2011).

A IN n° 62/2011, manteve basicamente os mesmos níveis dos parâmetros físico-químicos já estabelecidos na IN n° 51/2002, para leite cru refrigerado e para o leite pasteurizado, com exceção do índice crioscópico que passou a ter limites entre -0,550 °H e -0,530 °H (ou -0,531 °C e -0,512 °C). Em relação às análises microbiológicas, a IN n° 62/2011, alterada pela IN nº 31/2018, de 29 de junho de 2018, teve uma redução nos níveis máximos admitidos para CCS e CBT (CPP), sendo que a CCS passou de 7,5 x 10^5 para 4 x 10^5 e a CBT passou de 7 x 10^5 para de 1 x 10^5 (BRASIL, 2018).

Atualmente a IN n° 77/2018 (BRASIL, 2018), estabelece que devam ser realizadas periodicamente as análises que já eram regulamentadas pela IN n° 51/2002 e pela IN n° 62/2011: temperatura, índice crioscópico, teste do álcool/alizarol, acidez titulável, densidade relativa, gordura, proteína, neutralizantes de acidez e reconstituintes de densidade e índice crioscópico, e incluiu outras como: lactose anidra e extrato seco total (EST) ou sólidos totais (ST), pesquisas de substâncias conservadoras (como antibióticos e antimicrobianos) e resíduos de produtos de uso veterinário (BRASIL, 2018). Os níveis estabelecidos pela IN n° 76/2018 são os mesmos já definidos nas IN n° 51/2002 e IN n° 62/2011, com exceção do índice crioscópico que passou a ter o limite entre -0,555 °H e -0,530 °H (-0,536 °C e -0,512 °C).

A IN nº 55/2020, do MAPA, de 30 de setembro de 2020 (BRASIL, 2020), traz uma alteração à IN n° 76/2018 e estabelece a temperatura de transporte do leite das propriedades até o estabelecimento beneficiador deve ser de no máximo 5 °C, podendo ser de até 7 °C, quando o leite apresentar contagem microbiológica máxima de 3 x 10^5 UFC/mL (BRASIL, 2020).

Em 1996, com o objetivo de melhorar a qualidade do leite produzido no país, iniciou a elaboração do Programa Nacional de Melhoria de Qualidade do Leite (PNMQL), em uma parceria do MAPA com a Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) e as associações de classe que representavam as indústrias (LIMA *et al.,* 2020). Desde então, a qualidade do leite cru produzido no Brasil tem sido analisada pela RBQL (Rede Brasileira de Qualidade do Leite), composta atualmente por onze laboratórios oficiais, distribuídos em sete Estados (CBQL, 2020). Atualmente, o leite cru refrigerado da granja leiteira deve ser analisado em laboratórios da RBQL com frequência mínima quinzenal e as análises do leite transportado nos caminhões-tanque devem ser realizadas diariamente.

Os padrões atualmente em vigor, estabelecidos pela IN n° 76/2018, máximo de 3 x 10^5 UFC/mL (ou 300.000 UFC/mL) para CBT e 5 x 10^5 CS/mL (ou 500.000 CS/mL) para CCS (BRASIL, 2018), são muito mais rigorosos do que aqueles implantados na primeira fase do PNMQL, de 1 x 10^6 UFC/mL (ou 1.000.000 UFC/mL) para CBT e 1 x 10^6 CS/mL (ou 1.000.000 CS/mL) para CCS. Ainda assim, a produção brasileira é considerada pouco competitiva devido a baixa qualidade do leite e produtividade do rebanho leiteiro. A qualidade do leite cru no país parece não ter evoluído ainda tanto quanto o esperado,

levando a sucessivas revisões dos cronogramas de implementação de novos padrões de CBT e CCS (LIMA *et al.,* 2020).

2.2 Qualidade físico-química do leite

As análises físico-químicas do leite como índice crioscópico, densidade e a estabilidade ao alizarol são utilizadas principalmente para verificar fraudes no leite e a análise do índice de acidez está relacionada à presença de microrganismos no leite, sendo que esses microrganismos metabolizam os açúcares presentes modificando as características do leite e consequentemente comprometendo a sua qualidade.

Conforme a IN n° 76/2018, o índice crioscópico do leite precisa estar entre -0,530 ºH e -0,555 °H (ou -0,512 ºC e -0,536 ºC) (BRASIL, 2018). Essa análise é uma das mais utilizadas para determinar fraudes econômicas no leite. O índice crioscópico corresponde ao ponto de congelamento do leite e serve principalmente como indicador de adulteração no leite por adição de água (GASPAROTT *et al.*, 2020). A densidade relativa no leite precisa estar entre 1,028 e 1,034 a uma temperatura de 15 ºC (BRASIL, 2018). A densidade acima dos níveis estabelecidos pode indicar que houve desnatamento ou, ainda, que algum produto corretivo foi adicionado ao leite. Porém quando esse parâmetro está abaixo do estabelecido, indica que houve adição de água ou que existem problemas relacionados à saúde dos animais (SOUZA *et al.,* 2018).

A estabilidade ao alizarol é um teste rápido, realizado no leite cru refrigerado, que deverá apresentar um resultado negativo, observado quando ocorre a produção de coloração vermelho tijolo na amostra de leite. O alizarol é o teste utilizado para verificar a estabilidade térmica do leite, indicando ou não se pode ser pasteurizado. A análise consiste na adição de uma mistura de álcool e alizarina ao leite. A alizarina serve como indicador de pH, assumindo as cores amarela em meio ácido, e violeta em meio alcalino. O meio alcalino pode indicar a adição de soda cáustica e meio ácido indica proliferação de microrganismos. O leite que apresentar resultado insatisfatório pode gerar problemas para a indústria sendo, portanto, descartado (SANDOVAL; RIBEIRO, 2021).

Para Fagnani *et al.* (2016) o teste do alizarol é uma das provas indiretas da acidez no leite, entretanto, alguns fatores podem interferir na relação entre pH, ácido lático e número de microrganismos tornando esse teste não totalmente confiável. Um desses fatores é a presença de microrganismos psicrotróficos, que não metabolizam lactose em ácido lático, fazendo com que altas contagens bacterianas nem sempre sejam acompanhadas de acidez. Outro fator é o fenômeno do leite instável não ácido (LINA), que apresenta instabilidade na prova do álcool alizarol sem possuir acidez de origem microbiológica.

A acidez do leite pode ser alterada por diversos fatores como a raça, período de lactação, mastites, aguagem e alimentação. A análise de acidez é realizada para avaliar a possível presença de microrganismos que metabolizam a lactose, formando ácido lático e acidificando o leite, fornecendo um resultado quantitativo. Na prática, o que se mede é o volume de hidróxido de sódio necessário para neutralizar o ácido lático presente no leite. O resultado da titulação é expresso em gramas de ácido lático/100 mL de amostra ou % ácido lático (SANDOVAL; RIBEIRO, 2021). A legislação brasileira estabelece que a acidez do leite deve permanecer entre 0,14 e 0,18 g/ácido lático/100 mL (BRASIL, 2018). A refrigeração do leite cru reduz consideravelmente a multiplicação de microrganismos aeróbios mesófilos, principais responsáveis pelo processo de acidificação do leite cru, mas possibilita o desenvolvimento de

microrganismos psicrotróficos, capazes de se multiplicar em temperaturas de refrigeração, inferiores a 7 °C (MARIOTTO *et al.,* 2020).

Os elementos sólidos do leite representam entre 12% e 13%, e a água, aproximadamente 87%. Os principais elementos sólidos do leite são: lipídios (3,9%), proteínas (3,4%), lactose (4,8%), minerais (0,8%) e vitaminas. Esses elementos, suas distribuições e interações são determinantes para a estrutura, as propriedades funcionais e a aptidão do leite para o processamento (LEDUR *et al.*, 2020). As análises de composição do leite, definidas pela legislação, determinam o percentual de gordura, proteína, lactose, ESD e EST.

A gordura possui um valor mínimo estabelecido em 3,0 g/100g de leite (BRASIL, 2018) para o leite cru refrigerado e para o leite pasteurizado. A porcentagem de gordura tende a variar mais que os outros componentes e diferentes fatores exercem influência sobre esta variação, dentre eles: raça, alimentação, estação do ano, idade, estágio de lactação e fatores ambientais (STARIKOF *et al.*, 2016). Quanto maior a quantidade de fibras na dieta, maior será o teor de gordura e níveis abaixo do especificado pela legislação podem indicar adulteração do leite por adição de água ou desnate (FERRER *et al.,* 2018). A gordura do leite é caracterizada pela existência de aproximadamente 400 tipos de moléculas de triacilgliceróis com ácidos graxos e a sua estrutura é responsável pelo comportamento do leite durante o processo térmico por pasteurização ou UHT (ALI *et al.*, 2018).

Os níveis de proteína no leite também sofrem influência das estações, estágios de lactação, raças, estado de saúde e frações do leite e são proporcionais à quantidade de gordura, ou seja, quanto maior a porcentagem de gordura maior será a porcentagem de proteína. Foram relatadas mais de 3.100 proteínas no leite bovino, sendo que essa complexidade pode auxiliar na identificação de alterações celulares, moleculares e químicas de patologias da glândula mamária (MAITY *et al.*, 2020). Para Vargas *et al.* (2019), a baixa qualidade nutricional de alguns tipos de pastagens tropicais influenciam na diminuição da quantidade de proteínas no leite. As pastagens tropicais apresentam menores teores de proteína degradável no rúmen e carboidratos fermentáveis que as pastagens temperadas, o que desfavorece a formação de proteína microbiana em nível ruminal e diminui os valores de proteína total no leite. A legislação estabelece o teor mínimo de proteína total em 2,9 g/100g (BRASIL, 2018).

A lactose é o açúcar presente no leite e tem o teor mínimo estabelecido em 4,3 g/100g de leite (BRASIL, 2018). A lactose compreende o principal componente do EST e é responsável pelo desconforto quando consumido pelas pessoas com intolerância à lactose. Os valores de lactose no leite podem sofrer diminuição quando produzido por animais com diagnóstico de mastite. Essa redução pode estar relacionada a elementos indiretos da fisiopatologia da mastite no animal, uma vez que há relação de aumento de CCS com redução de teores de lactose no leite (DAMASCENO *et al.,* 2020).

Os termos sólidos totais (ST) ou extrato seco total (EST) englobam todos os componentes do leite exceto a água. Por sólidos não gordurosos (SNG) ou extrato seco desengordurado (ESD) compreendem-se todos os elementos do leite, menos a água e a gordura. Conforme a IN nº 76/2018, o teor mínimo de EST no leite deve ser de 11,4 g/100g e o teor mínimo de ESD deve ser de 8,4 g/100g (BRASIL, 2018). O EST pode variar de acordo com vários fatores, entre eles, a raça do animal, tipo de alimentação, estágio de lactação, sazonalidade, manejo do intervalo da ordenha ou estado de saúde do animal quando apresenta mastite (JIMÉNEZ *et al.*, 2021). Os níveis de ESD podem ser utilizados para a verificação de fraudes no leite, sendo que uma redução em seu nível pode estar atribuída a fraude por adição de água. A densidade podem identificar adulteração do leite por adição de água, porém caso a adição de água for acompanhada

de reconstituintes como o sal, amido ou açúcar e for realizada de forma equilibrada, a prova de densidade pode não ser capaz de detectá-la (MELO *et al.,* 2021).

2.3 Qualidade microbiológica do leite

A qualidade microbiológica do leite pode ser analisada a partir da contagem bacteriana total (CBT) e da contagem de células somáticas (CCS), ambas realizadas no leite cru refrigerado (BRASIL, 2018). Para o leite UHT, as análises microbiológicas podem incluir análises de *Staphylococcus* spp e *Salmonella* spp e a contagem de microrganismos mesófilos, tendo o valor máximo em 100 UFC/mL de mesófilos (BRASIL, 1997). Já para o leite pasteurizado, pode ser realizada a contagem de *Enterobacteriaceae* (BRASIL, 2018).

A CBT deve ser medida trimestralmente e não deverá exceder 300.000 UFC/mL no tanque individual e 900.000 UFC/mL no tanque comunitário, antes do seu processamento no estabelecimento beneficiador (BRASIL, 2018). A CBT indica a qualidade microbiológica do leite e a adoção de condições gerais de higiene e refrigeração, desde a obtenção do leite até o seu envio para a indústria. Várias etapas podem ser consideradas críticas na produção do leite acarretando o aumento da CBT, como por exemplo, o tipo de ordenha e sua falta de higiene. Leite com alta CBT pode provocar um impacto negativo em toda a cadeia produtiva, sendo responsável por problemas como: alterações no sabor e odor do leite, desvalorização pelas empresas que realizam o pagamento por qualidade, alterações no tempo de validade do leite *in natura* e dos produtos lácteos e, até mesmo, problemas de saúde pública (QUEIROZ *et al.*, 2019).

A vida útil do leite processado está diretamente relacionada à carga microbiana inicial no leite cru e à composição de sua microbiota. Um dos problemas enfrentados pelo Brasil é o comprometimento da qualidade do leite ainda na propriedade, pois este já sai com altas contagens de microrganismos aeróbios mesófilos. Enquanto nos Estados Unidos, União Europeia e Nova Zelândia a contagem desses microrganismos apresentam valores inferiores a 1×10^5 UFC/mL, no Brasil a legislação permite contagens três vezes maiores, com limite estabelecido em 3×10^5 UFC/mL (MARIOTTO *et al.*, 2020).

A contagem de células somáticas (CCS) é outro parâmetro amplamente utilizado para a avaliação da qualidade do leite cru refrigerado. A CCS deverá ser de no máximo 500.000 CS/mL (BRASIL, 2018). A CCS é utilizada como indicadora de mastite e tem sido utilizada para avaliar e monitorar a saúde da glândula mamária em rebanhos leiteiros (SILVA; ANTUNES, 2018). A mastite pode se apresentar nas formas clínica ou subclínica. A primeira apresenta sinais evidentes, como edema e aumento de temperatura do úbere, endurecimento, dor, grumos e pus no local e alterações das características do leite. Já a forma subclínica, apesar de não apresentar sinais visíveis de inflamação no úbere, é caracterizada pelo aumento no número de células somáticas (MESQUITA *et al.,* 2019).

As células somáticas são as células de defesa (leucócitos) do organismo. Essas células migram do sangue para o interior da glândula mamária com o objetivo de combater agentes infecciosos e compreendem de 80% a 98% do total das células presentes na glândula mamária e no leite, as células epiteliais dos alvéolos, compreendem o restante, de 2% a 20% do total (BRASIL; NICOLAU; SILVA, 2015).

Em vacas sadias são encontradas baixas CCS, geralmente menores que 50.000 células/mL de leite. Valores de CCS até 250.000 CS/mL não afetam a produção e a qualidade do leite, enquanto contagens acima de 250.000 – 300.000 CS/mL podem ser indicação de infecção bacteriana do úbere. O uso de

leite com alta CCS determina efeito negativo sobre o crescimento e metabolismo das culturas láticas, comprometendo a qualidade e causando a coagulação de produtos lácteos. Apesar do conhecimento existente, ainda não foi completamente explorado o efeito de leite com elevada CCS sobre os leites fermentados e produtos lácteos (FARIA *et al.,* 2020).

Para Auldist (2020), a inflamação mamária causada pela mastite provoca uma série de alterações físicas, microbiológicas e químicas no leite. Essas alterações produzem mudanças na composição química do leite e, como os diferentes componentes do leite têm propriedades funcionais diferentes, isso leva a mudanças nas propriedades de processamento do leite.

Além da CBT e da CCS outras análises podem ser realizadas para verificar a qualidade microbiológica do leite, como a contagem de microrganismos psicrotróficos. Esses microrganismos apresentam capacidade de produção de enzimas lipolíticas e proteolíticas termoestáveis, que mantêm a sua atividade enzimática após a pasteurização ou o tratamento UHT, influenciando diretamente na qualidade do produto final, diminuindo sua estabilidade e vida útil e alterando o sabor e odor do leite (MARIOTTO *et al.,* 2020).

3 CONSIDERAÇÕES FINAIS

A avaliação da qualidade físico-química e microbiológica do leite é de extrema importância para assegurar a saúde do consumidor, além de garantir um retorno financeiro e melhor renda ao produtor de leite. Ao longo dos anos observa-se um aumento na exigência dos parâmetros utilizados para a avaliação da qualidade do leite cru refrigerado e pasteurizado no Brasil. A legislação tornou-se mais restrita, diminuindo os níveis máximos permitidos principalmente para os parâmetros microbiológicos como a CCS e a CBT. O leite cru refrigerado de melhor qualidade é necessário para que se possa produzir leite processado e produtos lácteos de qualidade. O Brasil é um dos principais produtores mundiais de leite, porém necessita aprimorar ainda mais a legislação que regulamenta a produção e a qualidade de sua produção leiteira.

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ARTICLE
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Quality of bovine milk produced in Brazil - physical-chemical and microbiological parameters: an integrative review

Qualidade do leite bovino produzido no Brasil - parâmetros físico-químicos e microbiológicos: uma revisão integrativa

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ABSTRACT

Introduction: Milk is a rich and essential food to human health. The quality of milk can be influenced by several factors. **Objective:** To conduct an integrative review of scientific articles available on the Capes portal of journals, focused on making a diagnosis of milk quality through analysis of physical-chemical and/or microbiological parameters. **Method:** The descriptor used in the research was "milk quality" and to exclude from the search all studies that did not refer to bovine milk, "NOT" "human, maternal, buffalo, goat, goat, sheep" was typed. The following search mechanisms were also selected: "last ten years", "articles" and "any language", generating a total of 5,084 articles. Of this amount, 15 articles published in the period from 2012 to 2020 were selected. **Results:** The analysis of the articles allowed to infer that the physical- chemical aspects did not show significant changes in most of the analyzed samples; however, 93% of the articles showed microbiological changes in the milk and, therefore, decreasing of its quality. **Conclusions:** There is a need for the adoption of good farming and manufacturing practices, besides effective ways of storing collected milk to guarantee its quality, without compromising the health of the consumer and the financial return of the producer.

KEYWORDS: Milk; Analysis Methods; Food Quality; Cattle; Food Microbiology

RESUMO

Introdução: O leite é um alimento rico e essencial à saúde humana. A qualidade do leite produzido pode ser influenciada por diversos fatores. **Objetivo:** Realizar uma revisão integrativa de artigos científicos disponíveis no portal de periódicos da Capes, que tiveram como foco realizar um diagnóstico da qualidade do leite por meio de análises de parâmetros físico-químicos e/ou microbiológicos. **Método:** O descritor utilizado na pesquisa foi "qualidade do leite" e, para excluir da busca todos os estudos que não se referiam a leite bovino, digitou-se "NOT" "humano, materno, bubalino, cabra, caprino, ovino". Foram selecionados ainda os seguintes mecanismos de busca: "últimos dez anos", "artigos" e "qualquer idioma", gerando um total de 5.084 artigos. Desse montante, foram selecionados 15 artigos publicados no período de 2012 a 2020. **Resultados:** A análise dos artigos permitiu inferir que os aspectos físico-químicos não demonstraram alterações significativas na maior parte das amostras analisadas, porém 93% dos artigos demostraram alterações microbiológicas no leite e tendo, por isso, diminuição de sua qualidade. **Conclusões:** Mostra-se a necessidade de adoção de boas práticas agropecuárias e de fabricação, além de formas eficazes de armazenamento do leite coletado para garantir a sua qualidade, não comprometendo a saúde do consumidor e o retorno financeiro do produtor.

PALAVRAS-CHAVE: Leite; Métodos de Análises; Qualidade dos Alimentos; Bovinos; Microbiologia de Alimentos

INTRODUCTION

Brazil has dairy farming as one of its main economic activities and, according to the Food and Agriculture Organization of the United Nations[1], é o quarto produtor mundial de leite, it is the fourth world producer of milk, behind only the United States, India, and China. Dairy farming is practiced throughout Brazil, but there are producers of different technological and organizational levels, some from family farming or small cooperatives and others with properties of high technological level. This activity is important for the country, both in the social and economic context[2]. The milk production chain generates income and taxes, and dairy cattle is a link for the development of the primary sector, as well as having an important socioeconomic function[3].

In addition to the economic issue, through the generation of employment and income for the population, milk is still essential in the food supply[4] and can be considered one of the most complete foods[5], standing out for being a food of high nutritional value and being a source of proteins, lipids, sugars, minerals, and vitamins. In addition, it is necessary at all stages of human development, from birth to old age[6].

According to Martins et al.[7], the quality of the milk produced can be influenced by several factors, including those associated with the management, feeding, and genetic potential of the herds or those related to the collection and storage of milk, and refrigeration drastically reduces the multiplication of microorganisms in the milk.

The health of the mammary glands is another decisive factor in the quality of milk. During the milking process, for example, bacterial contamination can occur from the udder, the milker's hands, milking equipment, or poorly sanitized barrels and buckets. In these cases, the greatest contamination is by environmental microorganisms such as coliforms, particularly *Escherichia coli*. This contamination can also occur because of mastitis, which is a disease caused by both contagious pathogens and microorganisms from the environment[6]. Milk to be considered of good quality must have chemical, microbiological (total bacterial count - TBC), organoleptic, and somatic cell count (SCC) composition that meet the parameters required by law[8].

So that milk contamination does not occur, care such as hygiene of the milker, treatment of sick cows, cleaning, and daily disinfection of all equipment used in milking are essential. In addition, cooling the milk immediately after milking and collecting it in bulk are other important measures to ensure the microbiological quality of the milk, that is, the implementation of good practices in the stages of production and obtaining the milk, called good agricultural practices (GAP), is essential[9].

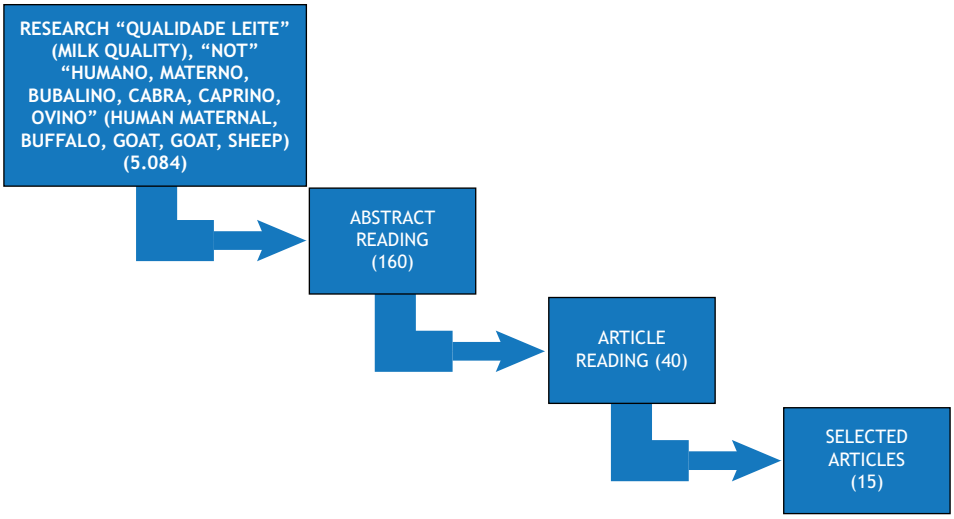
GAP consists of the production, processing, storage, transport, and distribution of raw materials, inputs, and agri-food products, maintaining all production links until they reach consumers. This provides a guarantee of quality and safety of milk quality, as well as adding value to the food production system and prevents possible contamination during the process of obtaining the product[9]. In addition, the Ministry of Agriculture, Livestock and Supply (MAPA) has milk quality monitoring programs, such as the *Programa Mais Leite Saudável* (PMLS) (More Healthy Milk Program, in English), which develops strategies to monitor the quality of milk produced in Brazil using tools such as the Brazilian Milk Quality and Monitoring System (SIMQL)[10].

This article aimed to carry out an integrative review of studies on the quality of bovine milk, *in natura* or processed, in which analyzes of physical-chemical parameters such as: acidity; density; percentage of fat, lactose, proteins, urea nitrogen; cryoscopic index; defatted dry extract (DDE) and total dry extract (TDE); and/or microbiological parameters such as: TBC, SCC, total and thermotolerant coliforms, *Salmonella* spp., mesophilic, psychrotrophic, and mastitic microorganisms such as *Staphylococcus* spp. Normative Instructions (NI) No. 76, of November 26, 2018[11], and No. 77, of November 26, 2018[12], of MAPA, regulate, respectively, the identity and quality characteristics that refrigerated raw milk, pasteurized milk, and type A pasteurized milk must present and the criteria and procedures for the production, packaging, conservation, transport, selection, and reception of milk.

METHOD

The journal platform of the Coordination for the Improvement of Higher Education Personnel (CAPES) was used. In the "advanced search" option, the descriptor "qualidade do leite" (milk quality) was typed for the search and as an exclusion criterion, "NOT" was typed "humano, materno, bubalino, cabra, caprino, ovino" (human, maternal, buffalo, goat, goat, sheep), in order to exclude the types of milk that were not bovine. In the field "data da publicação" (date of publication) we selected "últimos 10 anos" (last 10 years), in the field "tipo de material" (type of material), we selected "artigos" (articles) and in the field "idioma" (languages) we selected "qualquer idioma" (any language). A total of 5,084 articles were obtained. After reading the titles, 160 scientific articles were selected that mentioned the quality of bovine milk, which had their abstracts read. From this reading, 40 articles were selected for full reading. These articles included analyzes of the quality of bovine milk in different regions of Brazil. Of these, 15 articles from the last eight years (2012 to 2020) were selected. These articles presented analyzes of physical-chemical and/or microbiological parameters of milk and one of them (Ribeiro Neto et al.[13]) also presented a comparison of the influence of the periods of the year. The Figure shows the steps used to choose the articles.

To analyze the selected articles, the technique proposed by Bardin[14] called content analysis was used. From the reading of the selected articles, three categories were defined for the presentation of the results and discussion: milk collection and storage, physicochemical quality of milk, and microbiological quality of milk.



Source: Elaborated by the authors, 2020.

Figure. Steps used to choose the articles selected in this integrative review.

RESULTS AND DISCUSSION

The 15 selected articles presented results of physical-chemical and/or microbiological analyzes of bovine milk, according to the inclusion criteria. The Chart presents the selected articles, their objectives, and the main results obtained. The physical-chemical and microbiological parameters that were in disagreement with the legislation[11] were listed in more than 50% of the analyzed samples, in addition to the microorganisms found.

Milk collection and storage

The milk collection procedure and its transport need to follow international standards so that it is possible to compare analyzes from different laboratories. This promotes the diagnosis of milk quality on farms and in the industry, reaching consumers[28].

Samples were collected in sterilized flasks and kept in isothermal boxes in 40% of the analyzed articles, that is, six studies[15,17,19,20,24,26]. According to Leira et al.[29], low temperatures prevent or reduce the multiplication of most bacteria and decrease the activity of some degradative enzymes.

Four studies[13,16,22,23] used sterilized vials containing bronopol and azidiol-type preservatives. Preservatives are used to preserve the properties of the samples until they arrive at the laboratory for the analysis, and the vials must be opened only at the time of collection, being immediately closed afterwards[30]. Five studies[7,18,21,25,27], which corresponded to 33% of the analyzed articles, did not specify the form of collection and storage of milk samples.

Physicochemical quality of milk

Milk quality is evaluated by physical-chemical and microbiological parameters. Among the physical-chemical parameters we can mention analyzes such as: stability to alizarol, titratable acidity, relative density, and cryoscopic index. The composition of the milk is also an important indicator for its quality and, due to this, analyzes of the percentage of fat, protein, and lactose are carried out, in addition to TDE and DDE. These parameters reflect the health of the animals, the absence of chemical residues, and the conditions for obtaining and storing milk[31].

Of the 15 selected articles, ten (67%) presented data from the physicochemical analysis of milk. Bastos et al.[17], Molina et al.[18], Ribeiro Júnior et al.[21], and Silva et al.[25] performed acidity test, and Bastos et al.[17] and Silva et al.[25] found values as established in the legislation, from 0.14 g to 0.18 g of lactic acid/100 mL[11]. Molina et al.[18] and Ribeiro Junior et al.[21] found acidity values below the allowed in 61% and 54% of the samples, respectively, characterizing milk acidification. The acidity of milk is caused by the metabolism of microorganisms that cause the degradation of lactose, thus promoting an increase in the lactic acid content. Alkalinity can be attributed to mastitis or the addition of neutralizers[13]. Good quality milk should have a pH between 6.6 and 6.8, therefore slightly acidic[31].

Bastos et al.[17], Molina et al.[18], and Ribeiro Júnior et al.[21] performed relative density tests. The percentage of samples in disagreement for this parameter was, respectively, 10.3%, 10.0%, and 5.4%, thus, the three studies described values within the normal range for most samples.

Fresh, quality milk must have a relative density between 1.028 g/mL and 1.034 g/mL, at a temperature of 15°C[11]. The addition of water, in cases of fraud, reduces the density of the milk and the content of proteins, lactose, and mineral salts increase it[32].

Among the analyses, those of total solids or TDE were also performed by Bastos et al.[17], Martins et al.[7], Motta et al.[19], and Ribeiro Neto et al.[13] and none of the studies found values below the minimum reference content that is established by the current

Chart. Objectives of the selected articles, main results, and parameters in disagreement with the legislation[11].

Reference	Objectives	Main results and parameters in disagreement
Martins et al.[7]	Evaluate the microbiological and physicochemical quality and verify the occurrence of substances inhibiting microbial growth in raw milk from the individual and collective expansion tanks of a dairy industry located in the municipality of Rio Pomba, Minas Gerais.	Psychrotrophs, SCC, and presence of antimicrobials
Ribeiro Neto et al.[13]	To evaluate the quality of refrigerated raw milk under federal inspection of industries in several states of the Northeast region regarding chemical composition, SCC, and TBC.	SCC and TBC
Almeida et al.[15]	To characterize the refrigerated raw milk production system adopted in family farms in the municipalities of Bocaiúva, Francisco Sá, and Montes Claros, in the north of Minas Gerais, identifying the obstacles to milk production within the parameters established by the current legislation.	Presence of coliforms and *Staphylococcus* spp.: *S. aureus*, *S. intermedius*, *S. haemolyticus*, and *S. saprophyticus*
Angelis et al.[16]	It aimed to compare the TBC and SCC of raw milk obtained by manual and mechanized milking, and to measure the temperature of the milk at the time of reception at the dairy, in the city of Argirita, Minas Gerais.	TBC and SCC
Bastos et al.[17]	To evaluate the quality of refrigerated raw milk produced in family production units, in the south of Espírito Santo, to verify compliance with legal standards.	DDE and TBC, presence of antibiotics, cadmium, and lead
Molina et al.[18]	To evaluate the presence of foreign or fraudulent substances and the physicochemical and microbiological characteristics of the milk sold informally in the municipality of Itaqui, Rio Grande do Sul.	Acidity, TBC, SCC, and antibiotic residues
Motta et al.[19]	To investigate the main indicators of quality, nutritional constituents, presence of microorganisms, and detection of substances that inhibit bacterial growth in samples of informal cow's milk commercialized informally in the Southeast region of the state of São Paulo.	SCC and TBC Isolation of *Staphylococcus* spp., *Streptococcus* spp. and Enterobacteriaceae
Nascimento Neta et al.[20]	To evaluate the microbiological quality through the detection of spoilage and pathogenic bacteria in addition to the detection of antibiotic residues in refrigerated raw milk produced on family farms in the city of Alegre, Espírito Santo.	Presence of total coliforms and *Escherichia coli*
Ribeiro Júnior et al.[21]	Evaluate microbiological and physicochemical parameters of refrigerated raw milk produced in 99 properties in the region of Ivaiporã, Paraná, from August to October 2010.	TBC
Rigolin-Sá et al.[22]	To evaluate the presence of mastitis in cattle producing refrigerated raw milk, produced in 11 dairy farms in the southwest of Minas Gerais in the period 2012 and to verify compliance with the legislation (NI n° 62).	TBC and SCC. Presence of total and thermotolerant coliforms
Rosa et al.[23]	CCS, milk composition and urea nitrogen analyzed in order to verify the percentage of tank and individual samples of animals that met the parameters of the legislation (NI n° 51), in addition to indicating the best production system to ensure the best quality of milk in the central region of Rio Grande do Sul.	SCC
Sequetto et al.[24]	To evaluate the microbiological quality of refrigerated raw milk samples, stored in expansion tanks of rural properties in Zona da Mata Mineira, as well as the influence of types of milking and storage in community and individual tanks.	Presence of total coliforms and *Escherichia coli*, 40% mesophilic aerobic bacteria
Silva et al.[25]	To verify the quality of UHT milk from three brands, through physical-chemical and microbiological analyzes in Campos Gerais, Minas Gerais.	
Sola et al.[26]	To characterize the microbiological aspects related to the milk production of the Curraleiro Pé-Duro cattle herd, evaluating 226 samples of raw milk collected from January 2013 to January 2014, aiming at the search for *Salmonella* sp. in a rural property located in the state of Goiás.	Presence of *Salmonella* sp., *being S. heidelberg* and *S. schwarzengrund* most frequent
Reis et al.[27]	To carry out the diagnosis of 20 properties producing raw milk, in a family economy regime, aiming at the characterization of productive factors and their associations with aspects related to milk quality. All properties are located in the Alto Rio Grande micro-region, south of Minas Gerais.	SCC and TBC

Source: Elaborated by the authors, 2020.
SCC: Somatic cell count; TBC: Total bacterial count; DDE: Defatted dry extract; NI: Normative Instruction; UHT: Ultra High Temperature.

legislation of 11.4 g/100 g[11]. TDE or total solids can be understood as the sum of the concentration of all milk components, with the exception of water. On the other hand, non-fat solids (SNG) or DDE comprise all elements of milk, except water and fat, consisting of the difference between TDE and fat content[31].

Six articles[7,13,17,18,19,21] performed DDE or non-fat solids analysis. Bastos et al.[17], Molina et al.[18], and Motta et al.[19] found levels below the reference value that is established by current legislation, which is at least 8.4 g/100 g[11], for 85.0%, 61.9%, and 43.0% of samples, respectively.

The feeding of cattle is one of the main elements that influence the quality of milk, requiring diets with balanced nutritional values[29]. Of the articles analyzed, 53%, that is, eight articles[7,13,17,18,19,21,23,27] described results of analysis of the percentage of fat. In all studies, this parameter was within the established range, which is at least 3.0 g/100.0 g[11], for most samples.

Four of these studies[7,13,17,23] had all samples within the regularity and Molina et al.[18], Motta et al.[19], Reis et al.[27], and Ribeiro Junior et al.[21] reported 67.00%, 62.00%, 83.83%, and 75.00% of the samples in accordance with legislation, respectively. Ribeiro Neto et al.[13] observed a large variation of this parameter in their study, but this variation kept rates above the minimum limit defined by the legislation, with an average of 3.7 g/100 g. The percentage of fat in milk is positively influenced by the amount of fiber in the diet, that is, when there is a higher fat content, it means that there is greater availability of quality fiber in the diet of the herd[33]. Milk has an average fat concentration of 3.6%, however, in cases where the concentration is less than 2.0%, adulteration of this milk should be considered. Fat is one of the components that suffers the most adulteration, which may occur by adding water and/or skimming milk[18].

Seven studies[7,13,18,19,21,23,27] described results of percentage of milk protein. According to legislation, these levels must be at least 2.9 g/100 g[11]. Five studies[7,13,18,23,27] reported having all samples within the established and Motta et al.[19] and Ribeiro Junior et al.[21] reported 77.00% and 86.86% of samples in accordance with legislation, respectively. According to Leira et al.[29], the percentage of protein varies according to the breed and is proportional to the amount of fat present in the milk, that is, the greater the percentage of fat in the milk, the greater the protein content. For Ribeiro Neto et al.[13], the levels of fat and protein, and DDE were influenced in the analyzed periods of the year, with the levels of fat and protein being lower in the driest months of the year and DDE in the wettest periods.

However, when urea nitrogen levels were evaluated, Motta et al.[19] found levels below 10 mg/dL in 73.00% of the samples. Urea nitrogen does not have levels established by current legislation. According to Leão et al.[34], urea nitrogen has ideal values between 10 and 14 mg/dL, and these values are a consensus among several studies that sought to quantify a range in which this parameter would not have a negative effect on animals. There are several factors that alter the concentration of urea nitrogen in milk. Among them we can mention the diet, the production system, the season of the year, and the method of analysis, and a low protein diet can reduce the concentration of urea nitrogen in milk.

Four studies[7,13,21,23] also performed analyzes of lactose percentage and cryoscopic index, finding acceptable levels for these parameters. According to NI No. 76/2018[11], the minimum level of lactose in milk must be 4.3 g/100 g and the cryoscopic index must be between -0.512°C and -0.536°C. Lactose is the sugar in milk and comprises a good part of the total solids, while the cryoscopic index serves to identify fraud in milk. The freezing temperature of milk is lower than that of water due to dissolved substances, mainly lactose and mineral salts[31]. Silva et al.[25] and Molina et al.[18] carried out other analyzes to verify fraud in the milk, such as the presence of hydrogen peroxide and chlorides, with negative results.

Microbiological quality of milk

In order to have a parameter on the quality of milk produced on rural properties or processed by the industry, microbiological analyzes are necessary. According to MAPA's NI No. 76/2018[11], the necessary analyzes are TBC and SCC. The selected articles also presented analysis results for the research of mesophilic and psychrotrophic microorganisms, total coliforms, thermotolerant and *E. coli*, *Salmonella* spp., *Staphylococcus* spp., *Streptococus* spp. and fungi[7,13,16,18,19,22,23,27].

Somatic cells are present in milk and are made up of the sloughing cells of the secretory epithelium and the body's leukocytes, coming from the bloodstream, including monocytes, lymphocytes, neutrophils, and macrophages. An increase in this number may be an indicator of subclinical mastitis[6]. This analysis is used as an indirect diagnostic criterion for subclinical mastitis, and there are several factors that influence SCC in milk, but infection of the mammary gland is the cause of greater interference. Mastitis causes an increase in this number, due to the defense cells migrating from the blood to the site of infection, in order to fight the causative agent[35].

Ten articles[7,13,16,17,18,19,21,22,23,27], 67% of those evaluated in this review, presented SCC data. The results showed that in eight[7,13,16,18,19,22,23,27], that is, 80% of these studies, the values were above the limit established by the legislation[11], which is a maximum of 500,000 CS/mL, in more than 50% of the analyzed samples.

Eight studies[13,16,17,18,19,21,22,27] presented the results for TBC and all reported values above the limits established by the legislation, which is a maximum of 300,000 colony forming units (CFU)/mL[11], for most samples (more of 50%). The TBC refers to the total number of aerobic microorganisms, allowing the evaluation of milk quality from the moment of milking to its storage[6].

According to Martins et al.[7], the most used analysis to monitor the microbiological quality of raw milk is the standard count of aerobic mesophilic microorganisms on plates, which quantifies the number of viable cells of microorganisms present in raw milk. For Santos et al.[36], mesophiles are microorganisms that multiply rapidly when milk is not stored under refrigeration and psychrotrophs are microorganisms that multiply at lower temperatures, from 0°C to 7°C.

Of the selected articles, 35%, that is, five[7,15,17,20,24], presented results for counting mesophilic and psychrotrophic organisms and reported psychrotrophic levels above 10^4 CFU/mL for respectively 90%, 30%, 16%, 10%, and 10% of the analyzed samples. Mesophilic and psychrotrophic organisms do not have levels specified in current legislation, but levels from 10^5 CFU/mL are sufficient to cause losses in milk composition. The refrigeration process of collected raw milk favors the proliferation of microorganisms from the psychrotrophic group, capable of developing at temperatures below 7°C[7]. The psychrotrophs found in milk are of environmental origin and may come from the soil, water, vegetation, or from the teat/udder and from inadequately sanitized milking equipment. These microorganisms are destroyed by heat treatment, but their enzymes are resistant[37].

Other groups of important mesophilic organisms in milk analysis are total and thermotolerant coliforms[29]. Among the articles

analyzed in this integrative review, five of them[15,20,22,24,25] presented data from the analysis of microorganisms from the coliform group (total and/or thermotolerant) and *E. coli*. The results showed high contamination by this group of microorganisms in four of the studies carried out[15,20,22,24], and only for Silva et al.[25] the results were negative.

The presence of thermotolerant coliforms and *E. coli* is associated with materials of fecal origin and is an indicator of unsatisfactory hygienic conditions. These microorganisms in high numbers indicate lack of hygiene in milking and inadequate cleaning of equipment and utensils that come into contact with milk and contaminated water[9]. In the etiology of mastitis there are contagious and environmental microorganisms. The main contagious agents are *S. aureus* and *S. agalactie* and among the environmental ones, *E. coli*, *Klebsiella pneumoniae* among others[38]. It should be noted that Silva et al.[25] performed analyzes on Ultra HighTemperature (UHT) milk. Sterilization, by the UHT process, gives rise to the so-called long life milk and aims to obtain a bacteriologically sterile product[39], which explains the negative result in the analyses.

Among the articles analyzed in this review, three also showed data from analysis of mastogenic microorganisms such as *Staphylococcus* sp[15,19,20], and Motta et al.[19] also performed the analysis of enterobacteria, streptococci, and fungi. The results showed high contamination in the study by Nascimento Neta et al.[20], and the study by Almeida et al.[15] reported isolation of *Staphylococcus* spp. in 9.05% (36) of the total samples analyzed, with the identified species: *S. aureus* (52.80%), *S. intermedius* (5.60%), *S. haemolyticus* (19.40%), and *S. saprophyticus* (22.20%). Martins et al.[7], Bastos et al.[17], and Molina et al.[18] described the presence of antimicrobial substances in the milk analyzed.

The microorganisms found in milk, in addition to causing changes such as the degradation of fat, proteins, and carbohydrates, which makes the product unacceptable for consumption, can cause foodborne infections. One of the most common examples of agents causing these infections are the mesophilic microorganisms of the genus *Salmonella*, which cause intestinal disorders, in addition to vomiting and malaise[40]. The study by Sola et al.[26] presented results of analysis of *Salmonella* spp. finding six isolates in 226 milk samples of the Curraleiro Pé-Duro breed. Coliform microorganisms, *Salmonella* spp., as well as mastogenic ones such as *Staphylococcus* spp., do not show levels specified in current legislation, NI No. 76/2018[11]. The articles analyzed in this review used NI No. 62, of December 29, 2011[41], and NI No. 51, of September 18, 2002[42], />, which aimed to regulate the production, identity, and quality of A, B, C, raw, refrigerated, and pasteurized milk, in addition to collection and transport. These laws were repealed by the current legislation: NI No. 76/2018[11] and NI No. 77/2018[12].

CONCLUSIONS

The analysis of the articles in this integrative review showed that the physical-chemical parameters did not show significant changes in most of the samples analyzed in the studies in question. Regarding the microbiological parameters, 93% of the studies analyzed here showed microbiological alterations in the milk, thus reducing its quality. The exception occurs in a single study that analyzed milk sterilized by the UHT process. TBC, SCC, and counts of mesophiles and psychrotrophs outside the established standards, contamination by microorganisms of the coliform group, *Salmonella* spp., *Staphylococcus* spp.,fungi, and presence of antimicrobials in the analyzed samples were verified.

The adoption of GAP and manufacturing is important to remedy this contamination, as well as the education of producers regarding the hygienic-sanitary issues involved in the milking process. The form of milk storage is also essential to guarantee its quality, thus avoiding losses in milk quality, which also cause economic losses to the producer, as well as a risk to the health of the consumer population.

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Author's Contributions

Müller TM – Conception, planning (study design), acquisition, analysis, data interpretation, and writing of the work. Rempel CR – Conception, planning (study design), data interpretation, and writing of the work. All authors approved the final version of the work.

Conflict of Interests

The authors inform that there is no potential conflict of interest with peers and institutions, politicians, or financial in this study.

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Physicochemical and microbiological quality of bovine milk from Vale do Taquari in Rio Grande do Sul, Brazil

Qualidade físico-química e microbiológica do leite bovino do Vale do Taquari no Rio Grande do Sul, Brasil

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Abstract
The goal of the present study was to verify the quality of refrigerated raw milk from dairy properties and also of refrigerated raw milk, pasteurized and Ultra High Temperature (UHT) milk from industries in Vale do Taquari in Rio Grande do Sul, Brazil. Physicochemical, microbiological and milk composition analysis were carried out, as established by legislation, in addition to total and thermotolerant coliforms and psychrotrophic counts in the three types of milk and mesophiles count in pasteurized milk and UHT milk from the industries. The collections took place in two industries and 33 dairy properties. Regarding the properties, two (6%) had milk with acidity above that established by legislation and three (9%) had milk with a total bacterial count (TBC) above the established. The milk from industry 1 presented acidity, TBC and density out of the established standards. The two industries and 53.2% of the properties had milk with somatic cell counts (SCC) above that determined by legislation. The milk from the industries showed higher amounts of SCC, TBC, psychrotrophic and total and thermotolerant coliforms than the milk from the dairy properties, and the milk from industry 1 showed higher amounts than the industry 2, in the microbiological parameters.
Keywords: Physicochemical parameters; Microbiological parameters; Milk composition.

Resumo
O objetivo do presente estudo foi verificar a qualidade do leite cru refrigerado das propriedades produtoras de leite e do leite cru refrigerado, pasteurizado e *Ultra High Temperature* (UHT) das indústrias do Vale do Taquari no Rio Grande do Sul, Brasil. Foram realizadas análises de composição do leite, análises físico-químicas e análises microbiológicas, estabelecidas pela legislação, além de coliformes totais e termotolerantes, contagem de psicrotróficos nos três tipos de leite, contagem de mesófilos no leite pasteurizado e no leite UHT das indústrias. As coletas ocorreram em duas indústrias e 33 propriedades produtoras de leite. Em relação às propriedades, duas (6%) apresentaram leite com acidez acima do estabelecido pela legislação e três (9%) apresentaram leite com contagem bacteriana total (CBT) acima do estabelecido. O leite da indústria 1 apresentou acidez, CBT e densidade fora dos padrões estabelecidos. As duas indústrias e 53,2% das propriedades apresentaram leite com contagem de células somáticas (CCS) acima do determinado pela legislação. O leite das indústrias demonstrou maiores quantidades de CCS, CBT, psicrotróficos e coliformes totais e termotolerantes que o leite das propriedades produtoras de leite e o leite da indústria 1 apresentou maiores quantidades que a indústria 2, nos parâmetros microbiológicos.
Palavras-chave: Parâmetros físico-químicos; Parâmetros microbiológicos; Composição do leite.

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Introduction

Milk and dairy products are necessary foods, consumed daily and provide a valuable source of several macro and micronutrients [1]. Milk production in Brazil was 6.2 billion liters in the third quarter of 2021, with an amount of 25.3 billion in the same year. The state of Rio Grande do Sul (RS), with a production of 15.1 billion liters, is considered the second largest national milk producer [2]. The Vale do Taquari in RS is responsible for a large part of the state's milk production, and this activity is the basis of the economy of the small municipalities that compose it.

The quality of raw milk produced in Brazil must be analyzed by the Brazilian Milk Quality Network (RBQL)[3]. The parameters used for the diagnosis of milk quality include microbiological analysis, such as total bacterial count (TBC) for refrigerated raw milk and mesophilic microorganism count for pasteurized and UHT milk. In addition to physicochemical analysis, such as temperature, acidity, density, cryoscopic index, and analysis of milk composition, such as lactose, protein, fat, total dry extract (TDE) or total solids (TS) and defatted dry extract (DDE) or non-fat solids (NFS), for the three types of milk and, additionally, somatic cell count (SCC) and alizarol alcohol test, for refrigerated raw milk. These parameters and their limits are determined by the Ministry of Agriculture, Livestock and Supply (MAPA), through Normative Instructions N. 76/2018[4] and N. 77/2018[3], which determine the guidelines for refrigerated raw milk and for pasteurized milk and Ordinance N. 370/1997[5], which establishes the regulation of sterilized milk by the UHT process.

Considering the public health risks associated with the consumption of raw milk, heat treatment is applied to ensure the microbiological safety of dairy products[6]. The heat treatments used are pasteurization and UHT and aim to reduce the number of microorganisms to levels that are safe for consumer health[1]. In addition to the beneficiation processes, cooling the milk after milking and during transport at a temperature of up to 5 °C[7] is important for maintaining its quality, making the product viable for industrial processing. Low temperature reduces the microbial growth in the milk, but favors the proliferation of psychrotrophic microorganisms. Thus, counting psychrotrophic microorganisms is an important tool for evaluating the quality of produced milk. In addition to psychrotrophs, another important group used worldwide as indicators of hygienic conditions during milk processing are total and thermotolerant coliforms[8].

Thus, the information obtained through physicochemical, microbiological and milk composition analysis, determined by legislation and, additionally, counting of psychrotrophic microorganisms and analysis of total and thermotolerant coliforms, promote an accurate diagnosis of the quality of the produced milk. The goal of the present study was to verify the quality of refrigerated raw milk from dairy properties and of refrigerated, pasteurized and UHT raw milk from industries in Vale do Taquari in Rio Grande do Sul, Brazil, through physicochemical and microbiological analyzes, established by current legislation, in addition to the counting of mesophilic and psychrotrophic microorganisms and analysis of total and thermotolerant coliforms.

Material and methods

Study Area

The study was carried out in the region of Vale do Taquari (VT), Rio Grande do Sul, Brazil. Milk samples were collected from two dairy industries in the region and from 33 dairy properties, located in 33 of the 36 municipalities that compose the region. In the dairy industries, a total of six samples were collected: one of refrigerated raw milk from the tank truck, one of pasteurized milk and one of milk UHT, in each industry. In the properties, samples were collected from the milk cooler, one sample in each property, totaling 33 samples. The dairy industries were named I1 (Industry 1) and I2 (Industry 2) and the types of milk were given different abbreviations, being "Raw" for refrigerated raw milk, "Past." for pasteurized milk and "UHT" for UHT milk. The properties were named by the initial "P" followed by a number, being then determined from "P1" to "P33". The samples were collected in sterile flasks, stored and transported in isothermal boxes with ice. Analyzes took place within six hours after sample collection.

Physicochemical and milk composition analysis

In the refrigerated raw milk from the tank truck of the industries and properties, the milk composition analyzes followed the methods determined in Normative Instructions N. 77/2018, of MAPA[3]. The SCC analysis was performed using ISO 13366-2-IDF148-2:2006[9], and for this analysis a 40 mL bottle with Bronopol preservative was used to collect the samples. The other analyzes of milk composition: protein, lactose, total solids (TS) and non-fat solids (NFS) were carried out using ISO 9622-IDF141:2013[10].

To perform the alizarol test in the raw milk from the properties and the tank truck from the industries, a 100 mL beaker and 75% alizarol-alcohol was used, in which 10 mL of the alizarol-alcohol solution was mixed at 10 mL of milk homogenizing[11]. For processed milk from the industries, pasteurized and UHT, 1L of sample was collected and the norms used for the analysis of milk composition were: non-fat solids (NFS): according to the manual of official methods for the analysis of foods of animal origin of MAPA[12]; Total solids: ISO 6731-IDF

21:2010[13]; Lactose: ISO 22662-IDF 198:2007[14]; Lipids: NMKL 40:2005[15] and Total Protein: ISO 8968-1-IDF 20-1:2014[16].

The acidity and density analyzes, performed on the refrigerated raw milk from the properties and on the three types of milk from the industries (refrigerated raw from the tank truck, pasteurized and UHT), followed the same methodology. The acidity analysis was performed by titration, in which 10 mL of milk was pipetted into a 100 mL beaker, and 5 drops of 1% phenolphthalein were added. Sodium hydroxide (NaOH) 0.1N was diluted until a persistent pink color identical to the standard for approximately 30 s. The acidity was calculated as follows: Titratable acidity, % lactic acid = V. 0.09. N x 100/v, in which: V: corresponds to the volume of 0.1N NaOH solution spent in mL; v: is the sample volume in mL; 0.09: refers to the lactic acid conversion factor and N: is the normality of the 0.1N NaOH solution[12]. Density analysis was performed using the lactometer equipment, in which 500 mL of the sample was placed into a measuring cylinder, without creating foam, and the equipment was inserted to perform the reading[12]. The temperatures of all samples were measured using an Incoterm thermometer (model 5135) and were performed at the time of collection. All physicochemical and milk composition analyzes were performed in triplicate to ensure the reliability of the results.

Microbiological analyzes

The TBC was carried out on the refrigerated raw milk of the properties and on the refrigerated raw milk from the tank trucks of the industries and was performed according to the methodology recommended by ISO 21187-IDF196:2004[17], which is determined by Normative Instructions N. 77/2018[3]. The analysis of mesophilic microorganisms was carried out in pasteurized milk and in UHT milk from the industries. The analysis of psychrotrophic microorganisms and the analysis of total and thermotolerant coliforms were carried out in the refrigerated raw milk, both from the properties and from the industries, and in the pasteurized and UHT milks from the industries. All microbiological analyzes were performed in triplicate.

The analysis of mesophilic and psychrotrophic microorganisms followed the methodology recommended by the *Standard Methods for the examination of dairy products*[18]. Five decimal dilutions were performed in tubes containing 9 mL of 0.1% peptone. For the determination and quantification of mesophilic aerobic microorganisms, the pour plating method was used, in which the Petri dishes received 1 mL of dilutions 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, with approximately 20 mL of Plate Count Agar (PCA) (OXOID®).

The Petri dishes were incubated inverted at 36 ± 1 °C for 48 hours. For the determination and quantification of aerobic psychrotrophic microorganisms, the spread plate method was used, in which the surface of PCA agar (OXOID®) received 0.1 mL of the dilutions. The inverted plates were incubated at 7 °C for 10 days. The counting of microorganism colonies was performed using a colony counter and the results were expressed in CFU/mL (Colony Forming Units per mL).

The analysis of total and thermotolerant coliforms was performed using the Multiple Tube Technique, a method recommended by ISO 4831:2006 [19], 1mL of the sample was inoculated in a series of 3 tubes in Lauryl Sulfate Tryptose Broth (OXOID®) in test tubes containing inverted Durham tubes. A dilution was performed using 0.1% saline peptone solution, concentration 10^{-1} and 1 mL was added in a series of 3 tubes Lauryl Sulfate Tryptose Broth (OXOID®). The inoculated tubes were incubated at 30 ºC for 24 or 48 hours in a bacteriological incubator. The tubes with a positive presumptive reaction, evidenced by the production of gas, were then submitted to the confirmatory test in Brilliant Green Bile Lactose (2%) Broth (OXOID®). The tubes that showed gas formation in the Brilliant Green Bile 2% test were transferred to *Escherichia coli* broth (EC) and remained in a water bath for 48 hours at a temperature of 45 ± 0.2 ºC.

To check the milk quality, the levels found in the analyzes were compared with the limits defined by Normative Instruction N. 76/2018 of MAPA[4], for refrigerated and pasteurized raw milk and by Ordinance N. 370/1997 of MAPA[5], for UHT milk. The legislation does not establish levels of psychrotrophs and total and thermotolerant coliforms. The results were then compared with the limits established by authors in the area[20-8].

Data analysis

The data were tabulated using an Excel spreadsheet and the descriptive statistics were performed for quantitative data with the Bioestat 5.0 program, multivariate analysis of principal components (PCA) and correlation with the Past program. The results were compared: of the physicochemical and milk composition analyzes and the amounts of microorganisms found in the samples of the three types of milk (refrigerated raw, pasteurized and UHT), from the two industries analyzed in this study, the refrigerated raw milk from the dairy properties and the milk of the industrial tank truck.

Results and discussion

The results of the analysis of the parameters of milk composition (protein, lactose, fat, TS, NFS and SCC) and physicochemical parameters (temperature, acidity and density) show that the storage temperature in 31 of the 33 properties (93.93%) was lower than 5 °C, and only two properties (P9, P4) had a temperature higher

than this (Table 1). In property P9, the temperature measured at the time of collection was 7.8 °C. This is due to the fact that in this property the, collection occurred minutes after the milking, not allowing the milk to be sufficiently cooled in the cooler.

Table 1. Levels of physicochemical and milk composition parameters, average, standard deviation and standard error of refrigerated raw milk samples from dairy properties in Vale do Taquari - RS. ex. fat: excess fat - sample with a fat level around 10%; TS: Total solids; NFS: Non-fat solids

Unity	Temp. °C	Acidity g latic acid/100 mL	Density g/mL	Fat g/100g	Protein g/100g	Lactose g/100g	TS g/100g	NFS g/100g	SCC SC/mL
Levels	up to 5	0.14 to 0.18	1.028 to1.034	min.3.00	min. 2.90	min.4.30	min.11.40	min.8.40	up to500,000
P1	3.0	0.18	1.032	4.05	3.45	4.42	13.00	8.95	1,004,000
P2	3.6	0.15	1.033	3.64	2.94	4.45	12.04	8.40	459
P3	2.8	0.18	1.033	4.09	3.25	4.49	12.89	8.80	182
P4	5.1	0.18	1.033	4.09	3.42	4.46	13.17	9.08	1,812,000
P5	4.8	0.16	1.032	3.46	3.27	4.42	12.40	8.94	1,340,000
P6	3.7	0.15	1.031	3.57	3.14	4.49	12.45	8.88	333
P7	3.4	0.19	1.033	4.50	3.45	4.25	13.27	8.77	514
P8	3.7	0.16	1.031	3.67	2.89	4.45	12.12	8.45	159
P9	7.8	0.17	1.032	3.93	3.12	4.66	12.69	8.76	365
P10	3.4	0.15	1.033	3.62	3.07	4.45	12.22	8.60	909
P11	4.2	0.17	1.033	3.94	3.30	4.33	12.66	8.72	658
P12	4.4	0.16	1.031	3.85	3.29	4.59	12.79	8.94	404
P13	4.1	0.16	1.033	3.76	3.10	4.50	12.48	8.72	455
P14	4.2	0.19	1.033	3.65	3.18	4.44	12.40	8.75	1,180,000
P15	4.2	0.16	1.032	4.00	3.37	4.38	12.87	8.87	383
P16	4.1	0.16	1.033	3.86	3.37	4.55	12.78	8.92	365
P17	4.5	0.18	1.032	3.89	3.36	4.36	12.72	8.83	705
P18	4.8	0.15	1.030	4.35	3.11	4.22	12.80	8.45	1,489,000
P19	4.9	0.16	1.033	7.88	3.03	4.29	15.99	8.11	1,218,000
P20	4.2	0.18	1.033	4.48	3.80	4.37	13.76	9.28	1,290,000
P21	3.5	0.15	1.029	3.25	3.01	4.15	11.65	8.40	495
P22	3.4	0.20	1.032	3.70	3.61	4.30	12.81	9.11	894
P23	2.8	0.18	1.031	3.74	3.14	4.57	12.55	8.81	230
P24	3.9	0.15	1.031	3.69	3.10	4.45	12.36	8.67	467
P25	3.7	0.16	1.032	3.77	3.41	4.49	12.81	9.04	507
P26	4.1	0.18	1.030	4.42	3.47	4.25	13.32	8.90	883
P27	4.3	0.16	1.032	3.82	3.28	4.35	12.51	8.69	780
P28	4.2	0.17	1.032	3.66	3.01	4.51	12.22	8.56	133
P29	2.8	0.17	1.029	3.86	3.11	4.35	12.39	8.53	409
P30	1.8	0.18	1.032	4.02	3.31	4.62	12.95	8.93	551
P31	4.1	0.16	1.030	3.69	3.29	4.25	12.19	8.50	706
P32	4.2	0.17	1.030	8.11	2.59	4.30	15.98	7.87	190
P33	4.3	0.17	1.030	ex. fat	ex. fat	ex. fat	ex. fat	ex. fat	exc, fat
Average	4.04	0.17	1.031	4.14	3.23	4.41	12.87	8.73	670,906.25
Standart Deviation	1.03	0.00	1.26	1.12	0.29	0.00	0.80	0.38	425,317.13
Standart Error	0.18	0.00	0.22	0.19	0.05	0.00	0.14	0.06	75,186.15

Proper cooling of milk, on the farm and during transport, decreases the rate of bacterial growth, preventing the increase in bacterial counts, before the milk reaches milk collection centers or processing industries [21]. The physicochemical parameters of milk are very important for its acceptance by the consumer. Industry processes depend directly on these parameters to ensure a long shelf life for the product, adequate characteristics and nutritional benefits for the consumer. Parameters such as pH, oxidative stability and milk

composition are extremely important for the processing performed in dairy industries [22].

Regarding the industries, all samples of refrigerated raw milk and pasteurized milk had a temperature below the maximum allowed (Table 2), which is up to 5 °C, for refrigerated raw milk[7] and up to 4 °C, for pasteurized milk[4]. The pasteurized milk produced by industries in Vale do Taquari is originating from several herds, on several properties and transported under refrigeration[3]. The UHT milk from the industries is stored at room temperature, and the samples analyzed in this study had temperatures of 22.1 °C and 28 °C, in industries 1 and 2, respectively (Table 2).

Table 2. Levels of physicochemical, microbiological and milk composition parameters in samples of refrigerated raw milk, pasteurized and UHT milk from industries in Vale do Taquari - RS. *: for refrigerated raw milk; **: for pasteurized milk; ***: for UHT milk; nd: not determined. TS: Total solids; NFS: Non-fat solids.; ----: not performed

Parameters		Industry 1			Industry 2		
		Raw	Past.	UHT	Raw	Past.	UHT
Temperature (°C)	up to 5*; 4**	4.8	3.7	22.1	3.6	4.0	28.0
Acidity (g latic acid/100 mL)	0.14 to 0.18	0.67	0.20	0.24	0.18	0.17	0.18
Density (g/mL)	1.028 to 1.034	1.028	1.037	1.028	1.033	1.033	1.033
Fat (g/100g)	min. 3.00	4.37	3.80	3.00	3.80	3.20	3.00
Protein (g/100g)	min. 2.90	3.30	3.24	3.28	3.27	3.26	3.27
Lactose (g/100g)	min. 4.30	4.33	4.89	4.72	4.48	4.92	4.78
TS (g/100g)	min. 11.40*	13.08	12.42	11.50	12.59	12.02	11.92
NFS (g/100g)	min. 8.40 e 8.20***	8.71	8.60	8.50	8.79	8.80	8.90
SCC (SC/mL)	up to 500,000	1,079,000	------	------	638	------	------
TBC*/Mesophils (CFU/mL)	up to 900,000*up to 100***	4,599,000	9,7	0	466	510	0
Psychrotrophs (CFU/mL)	nd	10,000,000	80	0	10,000,000	40	0
Total coliforms (MPN/mL)	nd	110	0	0	110	0	0
Therm. Coliforms (MPN/mL)	nd	110	0	0	110	0	0

Regarding acidity, two properties, P7 and P22, showed the result above the limit established by current legislation (Table 1), with values of 0.19 and 0.20 g of lactic acid/100 mL, respectively [4]. According to Normative Instructions N. 76/2018 of MAPA[4], the acidity levels of refrigerated raw milk must remain between 0.14 and 0.18g of lactic acid/100 mL. The acidity analysis of the milks from the industries showed that industry 1 presented acidity above the maximum allowed level, in the three types of milk analyzed, raw refrigerated, pasteurized and UHT, with acidity levels of 0.67, 0.20 and 0 .24 g lactic acid/100mL, respectively (Table 2). The recommended acidity levels for pasteurized and UHT milk are also 0.14 to 0.18 g of lactic acid/100 mL, and are determined by Normative Instructions N. 76/2018 [4], if pasteurized milk, and by Ordinance 370/1997[5], if UHT milk. Acidity above the maximum allowed level indicates that failures in Good Agricultural Practices (GAP) may have occurred, or that the milk has been stored for a long time in the cooling tank[24]. Microorganisms present in milk ferment lactose, forming mainly lactic acid, which results in an increase in total acidity[24]. In industry 2, all physicochemical parameters and milk composition are in accordance with the parameters established by legislation.

The alizarol test, performed only on refrigerated raw milk, showed that clumps were formed in seven properties (P13, P15, P17, P27, P28, P29 and P32), which corresponds to 21.21% of the properties analyzed. The Alizarol test is one of the most used test to evaluate the milk quality and aims to verify the stability of milk proteins, when subjected to dehydration caused by alcohol, in order to estimate the stability of milk when subjected to heat treatment. In this way, it is possible to verify if the milk has sufficient thermal stability to support the processing processes of the industry, especially the UHT process.

The alizarol alcohol test, together with the milk acidity test, are used to identify unstable non-acid milk (UNAM) which is characterized as a set of alterations, in which the raw material presents acidity within normal standards, but they react positively to the alcohol test[25]. In this study, none of the properties that presented a positive alizarol test had acidity above the limit allowed in the samples, indicating the occurrence of UNAM.

In the refrigerated raw milk of the industries, the industry 1 showed a positive alizarol test for the refrigerated raw milk, with a yellowish color. The raw milk from the same industry showed acidity above the maximum allowed limit, being 0.67 g of lactic acid/100 mL. Pasteurized and UHT milk from industry 1 also had acidity above the allowed level, which can be explained by the very high acidity in refrigerated raw milk, almost four times higher than the maximum allowed.

Producing good quality raw milk is necessary to produce quality pasteurized and UHT milk. The relationships between practices on dairy properties, the composition, properties of raw milk and the quality of milk and dairy products are closely associated[26].

Regarding density, all samples from the properties were in accordance with the legislation, which should be from 1.028 g/mL to 1.033 g/mL[4]. Industry 1 showed density above the maximum allowed for pasteurized milk, which was 1.037 g/mL. Density is used as one of the parameters that seek to evaluate milk adulteration by adding water or constituents. In the case of fraud involving the removal of cream from milk, the density tends to increase, as the fat has a density of 0.930 g/mL. In the case of frauds by adding water, the density of the milk tends to decrease, and the density below the level can also indicate nutritional problems or health problems in the animal [24].

Regarding the averages of the physicochemical parameters and milk composition, the properties of Vale do Taquari showed higher averages of fat, TS and NFS than the dairy industries in the region and the industries showed a higher average of density than the properties analyzed. Acidity and lactose had equal averages, both in the industries and in the properties (Figure 1a). The temperature of refrigerated raw milk from the industries and properties showed a similar average. The average temperature of refrigerated raw milk from the industries was 4.20 °C and that of refrigerated raw milk from the properties was 4.04 °C. The pasteurized milk from the industries had an average temperature of 3.85 °C and UHT milk had an average of 25.05 °C. This is because the UHT milk from the industries is stored at room temperature.

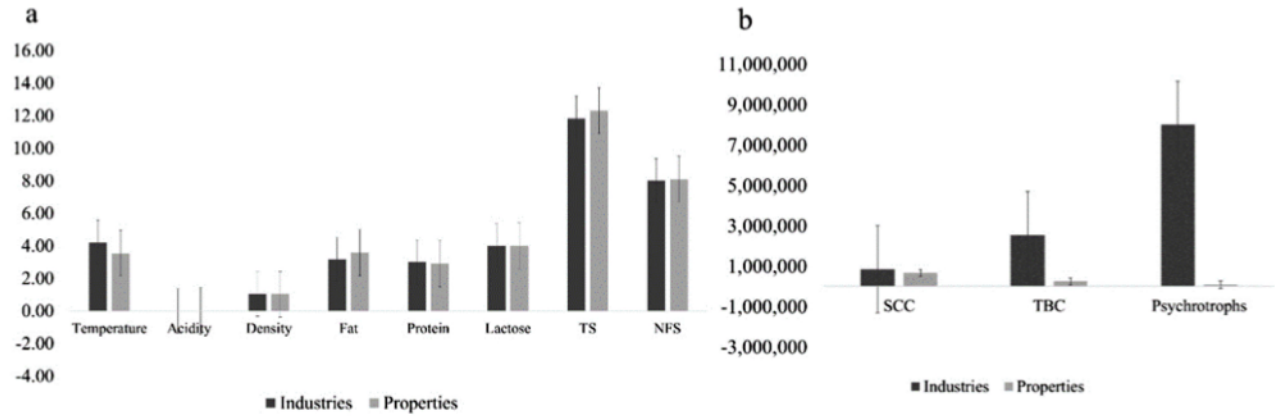

Figure 1. (a) Average of physicochemical parameters and milk composition of refrigerated raw milk samples from milk producing properties and samples of refrigerated, pasteurized and UHT raw milk from industries in Vale do Taquari - RS. For the average temperature of the industries, only refrigerated raw milk was considered. Figure with standard error. (b) Mean of SCC, TBC and psychrotrophic microorganisms found in samples of refrigerated raw milk from farms and dairy industries in Vale do Taquari-RS, with standard error.

The SCC analysis of the properties showed that 17 properties, which corresponds to 53.2% of the samples, had SCC levels above those allowed by the legislation, which is a maximum of 500,000 cells/mL[4]. Among these properties, seven (P1, P4, P5, P14, P8, P19 and P20), which corresponds to 21.2% of the samples, extrapolated the maximum measured by the method, with the SCC level established by estimation. The SCC of the properties ranged from 133,000 cells/mL, in property P28, to 1,812,000 cells/mL, in property P4. The average of the SCC in the properties was 670,906.25 cells/mL (Table 1). One of the samples, P33, had a fat content of around 10%, making it impossible for the method to measure the milk composition parameters (SCC, protein, lactose, ST and

NFS) and the fat itself accurately. This may be due to insufficient homogenization of the milk in the cooler.

According to the legislation, the analysis of SCC must be performed only on refrigerated raw milk[4]. Refrigerated raw milk from the dairy farm must be analyzed monthly and the milk transported in tank trucks must be performed daily[3].

The SCC rate in milk may be related to the immune reaction after infection of the mammary gland, called mastitis. Mastitis can be subclinical, when the number of leukocytes increases in the milk without apparent visual changes, or clinical when there are apparent changes in the milk. At times, this may be combined with signs in the udder or systemic clinical signs in the animal, which can be recognized by the farmer. A large amount of leukocytes presented as SCC and high TBC in milk can result in the production of enzymes that degrade its components, such as fats and proteins, reducing the quality of milk and dairy products. This also affects the shelf life of the product and reduces consumer acceptance[21]. In addition, the mastitic udder releases a large number of pathogenic microorganisms (including *Streptococcus* sp., *Staphylococcus* sp. and *Escherichia* sp.), which can contaminate bulk milk, becoming a public health problem[27].

When analyzing the relationship between the SCC and the other parameters of milk composition (fat, lactose, proteins, TS and NFS) of the properties, through Spearman's correlation analysis, it is possible to verify that the parameters presented a positive correlation, presenting greater amounts in properties with higher levels of SCC: fat (rs= 0.1712), protein (rs= 0.4185), NFS (rs= 0.2512) and TS (rs= 0.2594). The only exception was lactose (rs= -0.4689), which showed a negative correlation, with lower amounts in properties with higher amounts of SCC. This correlation is considered weak between SCC and fat, ST and NFS and moderates between SCC and fat and lactose. The correlation is significant between SCC and proteins and between SCC and lactose ($p<0.05$). According to Costa et al.[28], SCC above 200,000 SC/mL are indicative of subclinical mastitis, considering influences on milk components. In this study, only four properties (P32, P28, P8, P3), representing 12.12% of the samples, presented SCC below 200,000 SC/mL. Baggio and Montanhini[29], in their study, also reported a negative correlation between lactose and SCC, a fact that can be explained by the fact that inflammation of the mammary gland causes lesions in alveolar cells, leading to a decrease in lactose synthesis.

The SCC analysis performed in refrigerated raw milk from the tank truck of the industries, showed that both had the SCC above the maximum allowed by legislation[4], being 1,079,000 cells/mL in industry 1 and 638,000 cells/mL in industry 2 (Table 2). In industry 1, the amount of cells extrapolated the maximum amount obtained by the method, having its value established by estimate. The average SCC in the industries was 858,500 cells/mL, being higher than the average SCC of the properties, of 670,906.25 cells/mL (Figure 1b). The general average of SCC of milk in the VT region, taking into account the properties and the two industries, was 681,941.18 cells/mL. According to Ndahetuye et al.[21], increasing milk quality and safety worldwide is highly relevant, as regulations protecting consumer health require adherence to milk quality and safety guidelines, including low SCC.

The multivariate analysis of the principal components of the physicochemical parameters and milk composition show that component 1, temperature, explains 32.42% of the results, and component 2, acidity, explains 23.91%. Together, these two components explain 56.33% of the results. It is possible to observe an association between temperature, fat and TS and between acidity, density, protein, NFS and SCC (Figure 2).

The TBC analysis of the properties showed that 23 properties (69.70%) had counts up to 10^4 CFU/mL. Only three properties (P16, P17 and P33), which corresponds to 9% of the analyzed samples, presented TBC above the limit established by legislation (Table 3), which is up to 300,000 CFU/mL[4]. Sample P16 obtained a count of 596,000 CFU/mL. Sample P33, which was the same sample that showed large amount of fat, had a very high count of 1,559,000 CFU/mL. The bacterial count of milk-producing properties ranged from 8,000 CFU/mL (P6, P8, P9 and P30) to 4,534,000 CFU/mL (P17). In property P17, the result extrapolated the maximum measured by the method, is obtained by estimation. The TBC general average of the properties was 239,363 CFU/mL. The collection of milk by the tank truck, in the studied region, occurs every two days, and in 60% of the properties analyzed in this study, the collection of samples took place the following day or hours after the collection by the tank truck, in the case of properties that received him in the early hours of the morning. Thus, the cooler was almost empty, usually with the amount of one or two milkings in storage. The three properties (P16, P17 and P33) in which the TBC was above the allowed limit, are part of the 40% of the properties in which the sample collection took place hours before the tank truck collection (in properties where the collection took place in the early afternoon). Thus, in these properties, the cooler was full, at its maximum capacity, with the amount from several milkings, up to two days before. This suggests that the storage time of milk in the cooler may have an influence on the result of TBC found in dairy properties. Milk production in the properties analyzed in this study varies considerably, from 1,500 liters/month to 150,000 liters/month. However, most of the properties (85%) are made up of small producers, with an average of 12,000 liters/month.

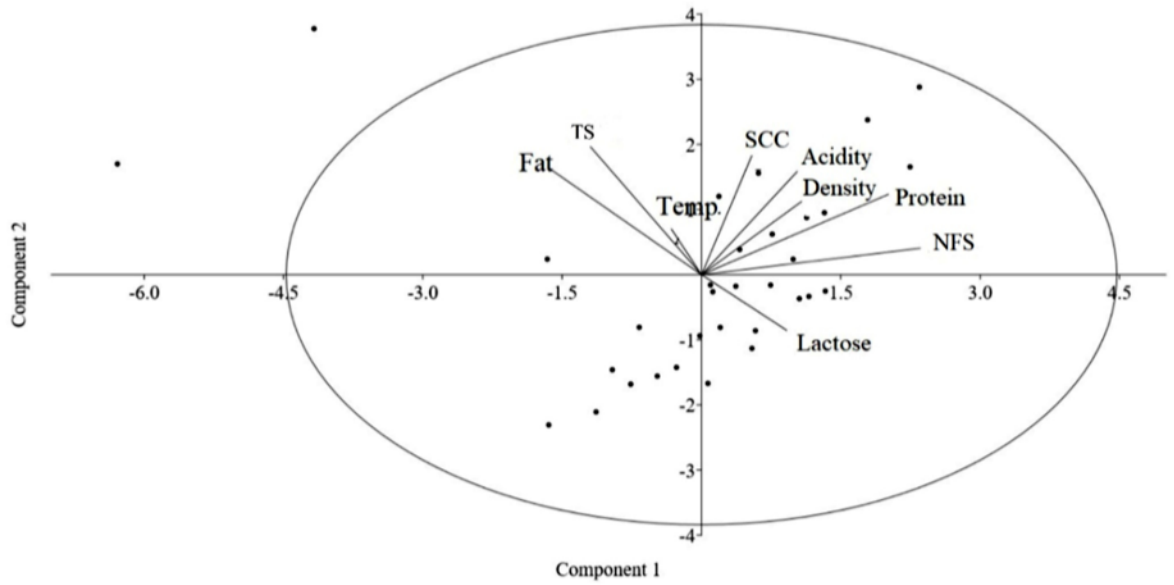

Figure 2. Ordination graphic about the multivariate analysis of the main components of the physicochemical parameters and milk composition of samples of refrigerated raw milk from dairy properties in Vale do Taquari - RS. TS: Total solids; NFS: Non-fat solids; Temp.: Temperature.

Table 3. Count of microorganisms of the analyzed microbiological parameters, average, deviation and standard error of refrigerated raw milk samples from dairy properties in Vale do Taquari - RS. ex. fat: excess fat; nd: not determined

Unity	TBC CFU/mL	Psychrotrophs CFU/mL	Total coliforms MPN/mL	Thermotolerant coliforms MPN/mL
Levels	up to 300,000	nd	nd	nd
P1	13	2	16	1
P2	15	9	21	0
P3	22	4	21	1
P4	113	2	110	21
P5	20	2,3	110	3
P6	8	5	14	1
P7	26000	1	14	3
P8	8	2	21	6
P9	8	1,5	110	110
P10	26	3	6	6
P11	37	4	21	2
P12	86	20	21	16
P13	52	70	21	2
P14	191	90	110	16
P15	50	1,8	3	3
P16	596	100	21	16
P17	4,534,000	2,000,000	110	15
P18	13	10	21	6
P19	15	10	110	6
P20	46	30	110	110
P21	122	2	16	2
P22	91	100	16	2
P23	21	500	21	1
P24	45	10	110	1
P25	15	200	1	1
P26	23	200	110	1
P27	21	900	2	1
P28	31	600	1	1
P29	11	100	6	2
P30	8	10	16	1
P31	24	10	2	1
P32	49	200	110	110
P33	1,559,000	20	16	2
Avarage	239,363.64	91,266.97	42.97	14.24
Standart Deviation	820,663.21	354,012.59	45.34	31.24
Starndart Error	142,589.12	61,625.68	7.89	5.43

Milk has ideal conditions for the growth of several types of microorganisms, including pathogens, due to its high water content, neutral pH and chemical composition[6]. The TBC is used to assess how much the milk production processes have affected its quality and safety. For Ndahuetuye et al.[21], TBC should be interpreted with caution, as different types of bacteria can contaminate milk, coming from different sources. These microorganisms proliferate in milk, as milk contains numerous nutrients for their growth and development.

The difference of TBC in the analyzed samples indicates that each property can be considered as a particular niche containing a dynamic microbiota. Farm management practices, milking systems and housing type have a large effect on TBC and bacterial composition of milk and may explain differences between the properties[27].

In the analyzes of TBC of refrigerated raw milk from the tank truck of the industries, it can be observed that industry 1 has TBC above the maximum amount allowed by current legislation (Table 2), which is up to 900,000 CFU/mL, for milk before industrial processing[4]. The amount of microorganisms in raw milk from industry 1 also extrapolated the maximum value measured by the method, having an estimate at 4,599,000 CFU/mL. Industry 2 presented TBC results within the limits of current legislation, with a TBC of 466,000 CFU/mL. The average TBC of the industries was 2,532,500 CFU/mL, higher than the average of the dairy properties, which was 239,363 CFU/mL (Figure 1b). The general average of refrigerated raw milk samples from Vale do Taquari was 1,387,431 CFU/mL. It should be noted that industry 1 also presented acidity in the samples of the three types of milk, positive alizarol test and SCC above the established limit, for refrigerated raw milk, and density out of the standards established by the legislation, for pasteurized milk.

Microbiological contamination of raw milk occurs mainly on the dairy farm, and the bacteria can be on milking equipment, milk storage places or inside the udder. Another fact is that the milk is not sterile inside the udder, not even in healthy cows, and these microorganisms can enter milk through the entero-mammary route[27]. The samples from the dairy properties in this study show great variation in microbiological parameters and SCC and, due to this, the standard deviation and standard error of the samples were extremely high (Table 1 and Table 3).

Pasteurized milk from industry 1 showed a mesophilic microorganism count of 9,700 CFU/mL. In industry 2, the count of mesophilic microorganisms was much lower, being 510 CFU/mL. The Normative Instructions N. 76/2018[4] does not establish the maximum amounts of mesophilic microorganisms for pasteurized milk. The UHT milk from both industries did not show colony growth, with the count of mesophilic microorganisms equal to zero (Table 2). According to Ordinance 370/1997, the amount of aerobic mesophiles must not exceed 100 CFU/mL in UHT milk[5].

Heat treatment in milk, pasteurization or UHT, is applied to ensure the microbiological safety of dairy products. In addition, it allows the inactivation of spoilage microorganisms and enzymes and, in this way, improves the quality and prolongs the shelf life of the products[6].

Pasteurization aims to reduce the number of any pathogenic microorganisms to a level where there is no significant danger to the health of the consuming public, still resulting in a shelf life of about ten days under refrigerated conditions. The UHT treatment, with temperatures of 135 °C for a few seconds, can guarantee a shelf life of up to six months, at room temperature, when kept in a closed bottle[1].

The analyzes of psychrotrophic microorganisms in refrigerated raw milk from dairy properties showed that most properties obtained counts up to 10^3 CFU/mL (13 properties) and 10^4 CFU/mL (9 properties), followed by 10^2 CFU/mL (6 properties) and 10^5 CFU/mL (3 properties). One property (P17) had a psychrotrophic count up to 10^6 CFU/mL, being 2,000,000 CFU/mL. The amount of psychrotrophic microorganisms ranged from only 10 CFU/mL, in property P30, to up to 2,000,000 CFU/mL, in property P17 (Table 3). The average number of psychrotrophic microorganisms from the properties was 91,266.97 CFU/mL, which is lower than the average TBC found in raw milk from dairy properties, which was 239,363.64 CFU/mL.

Property P17 presented the highest average in the count of psychrotrophic microorganisms, formation of clumps in the alizarol test, in addition to SCC and TBC above the limit established by legislation. Property P17 had its cooler at its maximum capacity, with the milk coming from several milkings, and the temperature was 4.5 °C at the time of collection, higher than the general average of the temperatures of the properties (4. 04 °C).

Psychrotrophic microorganisms are not considered an important component of the bovine udder microbiota and their presence in refrigerated raw milk probably occurs due to contamination during and/or after milking, with milking equipment, in most cases, being responsible for contamination. These microorganisms produce extracellular proteolytic and lipolytic enzymes during their growth in raw milk and these enzymes remain active after heat treatment. Psychrotrophic bacteria can grow considerably during cold storage, and both their metabolism and the production of extracellular enzymes can reduce the quality and shelf life of commercial milk and other dairy

products[30].

The amount of psychrotrophic microorganisms in raw milk from dairy farms (with an average of 91,266.97 CFU/mL) represents 38.12% of the TBC (with an average of 239,363.64 CFU/mL). Ribeiro Junior et al.[20], in their study, found a percentage of 78% of psychrotrophs in relation to the total bacterial count. For Mariotto et al.[31], milk with a good quality should not have a psychrotrophic count greater than 10% of the count of mesophilic microorganisms (or TBC), and this proportion gradually increases in contaminated milk.

The count of psychrotrophic microorganisms in the refrigerated raw milk from the industries was 10,000,000 CFU/mL, in industry 1 and 6,000,000 CFU/mL, in industry 2 (Table 2). The average amount of psychrotrophic microorganisms in raw milk from the analyzed industries was 8,000,000 CFU/mL, much higher than the average of psychrotrophic microorganisms in raw milk from dairy properties, which was 91,266.96 CFU/mL (Figure 1b). In relation to TBC, the average count of psychrotrophic microorganisms, of 8,000,000 CFU/mL, was more than three times higher than the average of TBC found in raw milk from the industries, 2,532,500 CFU/mL.

According to Paludetti, Kelly and Gleeson[32], the most common psychrotrophs found in raw milk during cold storage belong to the genus *Pseudomonas* sp. and milk with a psychrotrophic count greater than 5.0 x 10^6 CFU/mL should be rejected for processing, due to the possibility of production of enzymes by these microorganisms. Regarding the amounts of psychrotrophs found in the properties of this study, none exceeded this limit, and only one property (P17), representing 3% of the samples, had psychrotroph counts up to 10^6 CFU/mL. According to Ribeiro Junior et al.[20], who also obtained counts below 10^5 CFU/mL, in their study with raw milk, this evidences a high quality of milk produced on the properties. However, in relation to the industries, both presented counts between 10^6 CFU/mL (industry 2) and 10^7 CFU/mL (industry 1), exceeding the limit considered acceptable. According to the same author, the deterioration of milk by psychrotrophs is noticeable when the count reaches 10^6 CFU/mL.

The count of psychrotrophic microorganisms for pasteurized milk from the industries showed amounts above 10^3 CFU/mL in both industries, being 80,000 CFU/mL, in Industry 1, and 40,000 CFU/mL, in industry 2. The UHT milk from the industries did not show a count of psychrotrophic microorganisms (Table 2). The amount of psychrotrophic microorganisms in pasteurized milk from the industries (80,000 CFU/mL, in industry 1 and 40,000 CFU/mL, in industry 2) was much higher than the amount of mesophilic microorganisms of the same type of milk (9,700 CFU/mL, in the industry 1 and 510 CFU/mL, in industry 2). In industry 1, the amount of psychrotrophs is almost eight times greater than the amount of mesophiles and in industry 2, the amount of psychrotrophs is almost 80 times greater than the amount of mesophiles.

Although the quality of fluid milk and dairy products can be degraded through different mechanisms, chemical and/or microbial, microbial growth is predominant in spoilage. For pasteurized milk, one of the main causes of microbial spoilage of milk is post-pasteurization contamination (PPC) with gram-negative bacteria during processing[33]. A large number of bacteria can cause problems to the processed milk quality. These microorganisms are psychrotrophic, thermoduric, heat resistant and pathogenic. Spore-forming microorganisms and thermoduric enzymes produced by psychrotrophs are not destroyed by pasteurization and are common quality problems in milk and dairy products. Therefore, greater control of the initial microbial composition of raw milk is very important for the dairy industry in order to reduce or limit the economic losses caused by quality problems[27].

The amount of total coliforms in the properties ranged from 1 MPN/mL (two properties) to 110 MPN/mL (ten properties) and the amount of thermotolerant coliforms ranged from 1 MPN/mL (eleven properties) to 110 MPN/mL (three properties). The average amount of total coliforms in samples from dairy properties in Vale do Taquari was 42.97 MPN/mL and the average of thermotolerant coliforms was 14.24 MPN/mL. The ten properties (P4, P5, P9, P14, P17, P19, P20, P24, P26, P32) that presented total coliform counts at 110 MPN/mL, correspond to 33.03% of the analyzed samples. Of those, only three (P9, P20 and P32), 10% of the samples, presented the same amount for thermotolerant coliforms. Among these ten properties, seven (P4, P5, P14, P17, P19, P20, P26) had SCC above the limit established by legislation (Table 1). Of the three exceptions (P9, 924 and P32), two had SCC above 300,000 cells/mL, 365,000 (P9) and 467,000 (P24). Property P32, which also presented the amount of thermotolerant coliforms at 110 NMP/mL, showed low SCC, with a value of 190,000 cells/mL. Property P17, which showed positive SCC, TBC, alizarol and the highest count of psychrotrophs, as already mentioned, also showed higher amounts of total coliforms, showing failures in the hygiene of the property.

In industries, the amount of coliforms and thermotolerants in refrigerated raw milk was 110 NMP/mL for both, showing an average higher than the amount found in dairy properties (Figure 3a). Pasteurized milk and UHT milk from industries did not show the presence of coliform microorganisms.

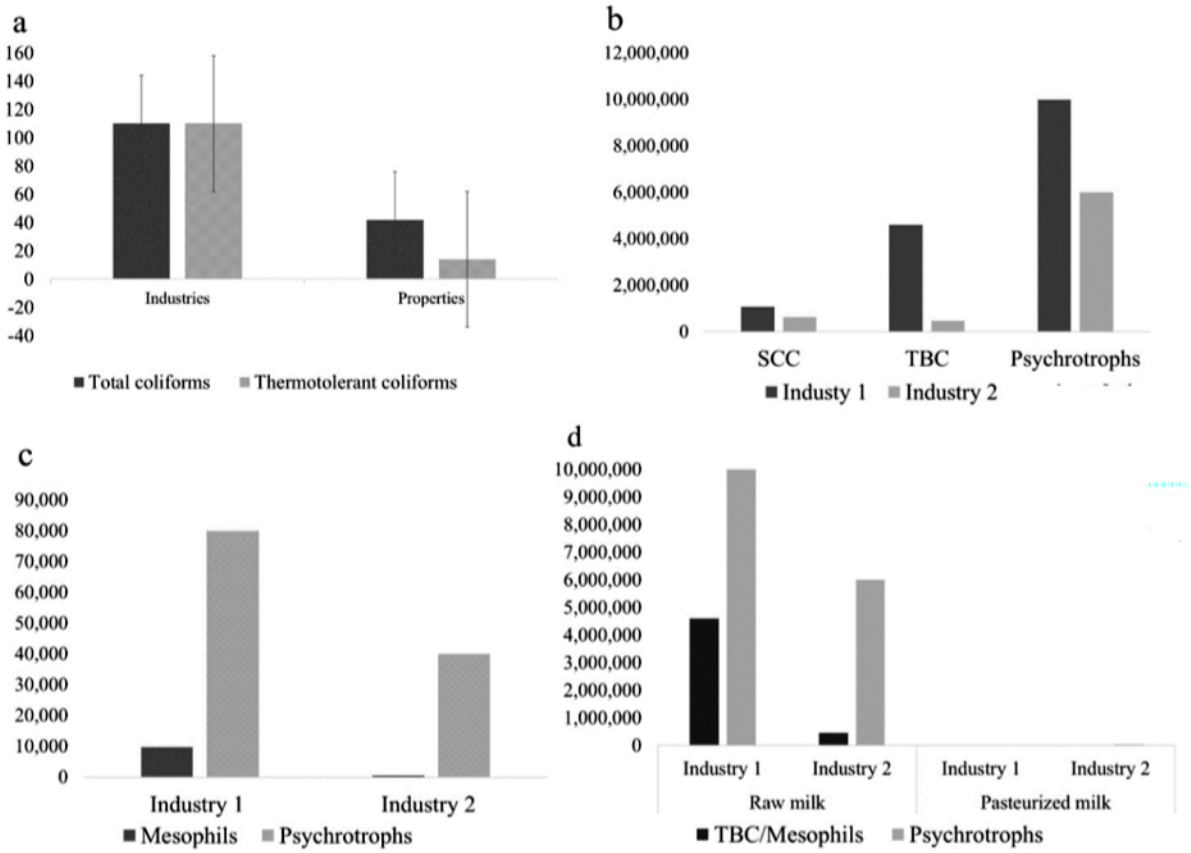

Figure 3. (a) Average amount of total coliforms and thermotolerant coliforms found in samples of refrigerated raw milk from industries and dairy properties in Vale do Taquari -RS and standard error; (b) Amount of SCC, TBC and psychrotrophic microorganisms found in refrigerated raw milk from industries 1 and 2 of Vale do Taquari – RS; (c) Quantity of mesophilic microorganisms and psychrotrophic microorganisms found in samples of pasteurized milk from industries 1 and 2 of Vale do Taquari – RS; (d) Amount of mesophilic microorganisms (or TBC) and psychrotrophic microorganisms found in refrigerated raw milk and pasteurized milk from industries in Vale do Taquari – RS.

The use of indicator bacteria, such as the group of total and thermotolerant coliforms, had as its initial concept the investigation of fecal contamination, later serving as an indirect indicator of pathogens. However, several studies over the years have shown a lack of correlation between the presence of indicators and pathogens in the samples. Thus, these indicators are currently used as sanitation indicators. Coliform bacteria can be found in low amounts, up to 100 CFU/mL, in refrigerated raw milk with good quality. However, the presence of large quantities has been mainly attributed to unsanitary processing conditions. Total and thermotolerant coliforms are not inherent microflora in raw milk and are introduced from the environment, udder and milking equipment[8]. In cases of *Escherichia. coli* in high numbers, foodborne outbreaks may occur[21].

The fact that refrigerated raw milk from industries has higher amounts of TBC, psychrotrophs and total and thermotolerant coliforms than refrigerated raw milk from dairy properties in Vale do Taquari, can be explained by the milk storage time, which can cause the proliferation of microorganisms, mainly psychrotrophic, which multiply at low temperatures. About the microorganisms of the coliform group, the contamination can come from the environment, at the time of collection of milk by the tank truck. The higher amount of SCC can be explained through the fact that the milk from the tank truck comes from several properties, which results in the mixing of milk with high SCC and milk with acceptable levels, causing a general increase in SCC in the milk of the tank truck.

When comparing the amount of SCC, TBC, mesophiles, psychrotrophs and total and thermotolerant coliforms of the two analyzed industries, it can be seen that industry 1 had the highest amount of SCC, TBC and psychrotrophs (Figure 3b), in refrigerated raw milk and

higher quantity of mesophiles and psychrotrophs in pasteurized milk (Figure 3c), in relation to industry 2. Thus, the milk from industry 2 has a higher microbiological quality than the milk from industry 1. Industry 1 needs greater attention in the storage and processing of milk, in addition to checking for possible post-processing contamination. The counts of psychrotrophs and SCC are 40% and 70%, respectively, higher compared to industry 2. The TBC of industry 1 is 10 times higher than the TBC of industry 2. Relative to pasteurized milk, the mesophilic count is 19 times higher in Industry 1 than in Industry 2, and the psychrotrophic count is exactly double the count found in Industry 2.

Despite this, it is possible to verify the reduction of TBC and the number of mesophilic and psychrotrophic microorganisms from refrigerated raw milk from the industries related to pasteurized and UHT milks. This indicates that, in general, the beneficiation processes of the industries have been effective (Figure 3d) in the reduction of microorganisms. This reduction reaches 100% of refrigerated raw milk compared to UHT milk. as it did not show the growth of mesophiles, psychrotrophs or the presence of total and thermotolerant coliforms. The pasteurized milk from the industries also did not present microorganisms of the coliform group, reinforcing the efficiency of this thermal process in the elimination of this group of microorganisms.

Conclusion

The physicochemical analyzes showed that two properties presented acidity above that established by the legislation for refrigerated raw milk. The milk composition analyzes showed that all properties and industries were in accordance with the established for lactose, protein, fat, ST and NFS, with the exception of property 17, which showed excess fat. The SCC was above the established limit for 53.2% of the properties and for the two analyzed industries. Microbiological analyzes showed that three properties and industry 1 had TBC above the established limit. Industry 1 also presented acidity above the allowed in the three types of milk, raw refrigerated, pasteurized and UHT, and density above the established for pasteurized milk. The industries showed higher amounts of SCC, TBC, psychrotrophs and total and thermotolerant coliforms than the dairy properties of Vale do Taquari. The raw milk from the properties showed higher quality compared to the refrigerated raw milk found in industrial tank trucks, a fact that can be caused by failures in cooling or prolonged storage periods. The refrigerated and pasteurized raw milk from industry 2 showed a higher microbiological and physicochemical quality than the refrigerated and pasteurized raw milk from industry 1. The evaluation of the quality of the milk produced is extremely important for the identification of possible failures in the production process, being essential for the implementation of improvements in all stages of the production chain.

Conflict of interests

The authors declare no conflict of interest

Author contributions

Conceptualization: T. Müller, M.J.Maciel and C. Rempel. *Data curation*: T. Müller. *Formal analysis*: T. Müller. *Investigation*: T. Müller. *Methodology*: M.J.Maciel. *Resources*: M.J.Maciel and C. Rempel. *Project management*: C. Rempel. *Validation*: T. Müller. *Visualization:* T. Müller. *Supervision*: M.J.Maciel and C. Rempel. *Writing (original draft):* T. Müller. Writing (review and editing): M.J.Maciel and C. Rempel.

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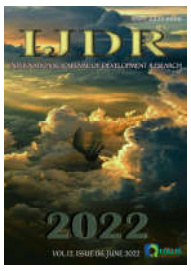

RESEARCH ARTICLE OPEN ACCESS

QUALITY OF REFRIGERATED, PASTEURIZED AND STERILIZED RAW BOVINE MILK FROM INDUSTRIES IN VALE DO TAQUARI – RS

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ABSTRACT

Milk is a food of animal origin, rich in nutrients and which, due to its nutritional properties, is a matrix for the growth of microorganisms. Physicochemical, microbiological and microbiome analysis in milk allow an accurate diagnosis of its quality. The aim of the present study was to evaluate the quality of refrigerated, pasteurized and sterilized raw milk from dairy industries in Vale do Taquari, Rio Grande do Sul, Brazil. Physicochemical and microbiological analyzes were performed, established by Brazilian legislation and the analysis of psychrotrophic microorganisms and total and thermotolerant coliforms. In addition, the microbiome was analyzed through high-throughput sequencing of the 16S rRNA gene. Both industries had somatic cell counts (SCC) above the limit established for refrigerated raw milk and psychrotrophic levels higher than those of mesophiles. Industry 1 presented acidity above the limit in the three types of milk, total bacterial count (TBC) and density for refrigerated raw milk and for pasteurized milk, respectively. The samples presented a wide diversity of genera, composed of psychrotolerant (*Kurthia*, *Acinetobacter*, *Viridibacillus*), biofilm formers (*Pseudomonas*), mastogenic (*Streptococcus*) and lactic acid (*Lactococcus*), in addition to genera considered harmful (*Escherichia*, *Citrobacter*, *Aeromonas* and *Enterobacter*).

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INTRODUCTION

Milk is an example of a nutrient-rich food of animal origin, which contains lipids, proteins (casein), carbohydrates (lactose), amino acids, vitamins and minerals (calcium), and which has several dietary benefits for humans. Due to all these nutritional properties, milk is also a growth matrix for a wide variety of spoilage and/or potentially pathogenic microorganisms (Lindsay *et al*., 2021). Bacterial contaminants in milk are usually animal skin, feed, air, soil, and milking equipment (Elegbeleye & Buys, 2022). The production of this drink represents an important contribution to the economy and social development, as around 150 million families work in milk production worldwide. Most dairy farmers use this activity for their subsistence and are small farmers residing in developing countries (Fao, 2021). Milk production in Brazil was 25.3 billion liters during the year 2021, and in the third quarter this production reached 6.2 billion liters. Rio Grande do Sul is the second largest national milk producer, with a production of 15.1 billion (IBGE, 2021).

Vale do Taquari, a region located in the central part of the state, is responsible for a large part of the state's milk production, and this activity is the basis of the economy of the small municipalities that comprise it. The quality of milk is influenced by several factors ranging from production on milk-producing properties to transport and processing carried out by the industry. The parameters used for the diagnosis of milk quality include analysis of the composition (lactose, protein, fat, total dry extract or total solids and defatted dry extract or non-fat solids), physical-chemical analysis (temperature, acidity, density and cryoscopic) and microbiological analyses, such as total bacterial count (TBC) or mesophilic microorganism count, for the three types of milk (refrigerated raw, pasteurized and sterilized milk) and additionally, alizarol test and somatic cell count (SCC) for refrigerated raw milk.These parameters and their limits are determined by current legislation, Normative Instruction (NI) No. 76/2018 (Brazil, 2018a), NI No. 77/2018 (Brazil, 2018b) and Ordinance No. 370/1997 (Brazil, 1997), from the Ministry of Agriculture, Livestock and Supply (MAPA). NI No. 76/2018 provides information on the identity and quality characteristics that refrigerated

raw milk, pasteurized milk and type A pasteurized milk must present (Brazil, 2018a). IN No. 77/2018 establishes the criteria and procedures for the production, packaging, conservation, transport, selection and reception of raw milk in establishments registered with the official inspection service (Brazil, 2018b), and Ordinance No. 370/1997 regulates the identity and quality of milk sterilized by the Ultra High Temperature (UHT) process (Brazil, 1997).One of the ways used to reduce the microbial growth of milk, causing its degradation, is the cooling right after milking and during transport. This cooling must occur at a temperature of up to 5 °C (Brazil, 2020) and remain below this temperature until reaching the dairy industry, where it will undergo processing. The decrease in temperature reduces bacterial proliferation, however, favors the proliferation of psychrotrophic microorganisms. Psychrotrophic microorganisms can produce heat-resistant proteolytic and lipolytic enzymes that remain active after heat treatment, potentially affecting the quality and shelf life of milk and dairy products. Thus, it is necessary to investigate the psychrotrophic bacteria existing in milk in order to control contamination and proliferation from its source (Yang *et al*., 2020).

In addition to the analysis of psychrotrophic microorganisms, the analysis of total and thermotolerant coliforms in milk plays an important role in the dairy industry, as these microorganisms are often used as indicators of hygiene in the milk production process (Masiello *et al*., 2016). Another current tool that has been used to identify the quality of milk produced is high-throughput sequencing, which provides detailed and valuable information about the microbial community in milk and dairy products in general.High-throughput sequencing enriches the understanding of the role of microorganisms in milk and dairy products (You *et al*., 2022). In this way, the information obtained through conventional physical-chemical and microbiological analyses, together with genetic sequencing, promote an accurate diagnosis of the quality of the milk produced, being also a tool for the improvement of the processing processes used in the dairy industry. Current Brazilian legislation does not specify acceptable levels of psychrotrophic microorganisms and total and thermotolerant coliforms and does not regulate the analysis of data obtained by sequencing. The objective of the present study was to evaluate the quality of refrigerated raw milk from tank trucks, pasteurized milk and milk sterilized by the UHT process, from dairy industries in Vale do Taquari - RS, through physical-chemical and microbiological analyzes established by current legislation, in addition to counting psychrotrophic microorganisms, analysis of total and thermotolerant coliforms and the microbiome, through high-throughput sequencing of the 16S rRNA gene.

MATERIALS AND METHODS

Six samples were collected in two industries, in two cities in Vale do Taquari - RS, one of refrigerated raw milk from the tank truck, one of pasteurized milk and one of milk sterilized by the UHT process in each of the industries. The industries were named "I1" for Industry 1 and "I2" for Industry 2, and the types of milk had their abbreviated names, being "Raw" for refrigerated raw milk, "Past.", for pasteurized milk and "Ster. ", for sterilized milk.Samples were collected using sterilized plastic bottles, and all hygiene precautions were followed. The samples were placed in a styrofoam box with ice, kept at a temperature below 7 °C. Physicochemical, microbiological and milk composition analyzes were performed up to 10 hours after sample collection and sequencing up to 24 hours after collection.

Molecular Analysis: The identification of microorganism genera was performed using high-performance sequencing of the V3/V4 regions of the 16S rRNA gene. The primers for the V3-V4 region of the 16S rRNA gene were: 341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). PCR reactions were performed in triplicates, with the conditions: 95 °C for 5 min, 25 cycles of 95 °C for 45 s, 55 °C for 30 s and 72 °C for 45 s and a final extension of 72 °C for 2 min. The MiSeq Sequencing System equipment (Illumina Inc., USA) was used to sequence the genomic libraries. For single-end sequencing, the V2 kit with 300 cycles was used. The sequences were analyzed using the Sentinel pipeline. In the Sentinel pipeline, fastq files are evaluated for Phred quality (QP) using the FastQC v.0.11.8 program (Andrews, 2010). Therefore, the fastq files were submitted to low quality primers and sequence trimming (Phred< 20). The software used for this purpose was built in Python v.3.6, which is inspired by the features of the BioPython project (Cock *et al*. 2009). For paired-end data, before the trimming step, two pairs of files (R1 and R2) were merged into one file using pandaseq v.2.11. Clusters with abundances less than two were removed from the analysis, as such structures are usually related to chimera sequences (Smyth *et al*., 2010). Taxonomic identifications were performed with BLASTn v.2.6.0 (Altschul *et al*., 1990), using a proprietary or public database as a reference.

Physicochemical and milk composition analysis: Sample temperatures were measured using an Incoterm thermometer (model 5135) at the time of collection. In refrigerated raw milk, the analyzes of milk composition: protein, lactose, total dry extract (TDE) and defatted dry extract (DDE) were carried out using ISO 9622-IDF141:2013 (ISO, 2013). The SCC analysis was performed using ISO 13366-2-IDF148-2:2006 (ISO, 2006), and for this analysis a 40 mL bottle with Bronopol preservative was used to collect the samples. To perform the alizarol test, a 10 mL beaker and 75% alizarol-alcohol was used. 10 mL of the alcohol-alizarol solution was mixed with 10 mL of milk and homogenized (Gasparotto *et al*., 2020). The rules used for the composition of milk in processed milk (pasteurized and sterilized) were: defatted dry extract, according to the manual of official methods for analyzing foods of animal origin by MAPA (Brazil, 2019); Total dry extract: ISO 6731-IDF 21:2010 (ISO, 2010); Lactose: ISO 22662-IDF 198:2007 (ISO,2007); Lipids: NMKL 40:2005 (NMKL,2005) and Total Protein: ISO 8968-1-IDF 20-1:2014 (ISO,2014). 1 liter of sample was collected to perform these analyses. The acidity and density analyzes followed the same methodology in the three types of milk. The acidity analysis was performed by titration, in which 10 mL of milk was pipetted into a 100 mL beaker, and 5 drops of 1% phenolphthalein were added. Sodium hydroxide (NaOH) 0.1N was then diluted until a persistent pink color identical to the standard for approximately 30s. The acidity was calculated as follows: Titratable acidity, % lactic acid = V. 0.09. N x 100/v, in which: V: corresponds to the volume of 0.1N NaOH solution spent in the solution in mL; v: is the sample volume in mL; 0.09: refers to the lactic acid conversion factor and N: is the normality of the 0.1N NaOH solution (Brazil, 2019). Density analysis was performed using a thermolacton densimeter equipment, in which 500 mL of the sample was poured into a beaker, without creating foam, and the equipment was inserted to perform the reading (Brazil, 2019). All analyzes were performed in triplicate.

Microbiological analyzes: The TBC analyzes were carried out according to the methodology recommended by ISO 21187-IDF196:2004 (ISO, 2004). The analyzes of mesophilic and psychrotrophic microorganisms were carried out using the methodology described in the Standard Methods for the examination of dairy products (Apha, 2004). For the determination and quantification of aerobic mesophilic microorganisms, the decimal dilution methodology was used, in which 1 mL of the sample was pipetted, transferring it to a tube containing 9 mL of 0.1% peptone. From this dilution, decimal dilutions 100, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were made. Petri dishes received 1 mL of the dilutions, with approximately 20 mL of Plate Count Agar (PCA) agar (OXOID®), using the depth plating method, with inverted plates incubated at 36 ± 1 ºC for 48 hours. For the determination and quantification of aerobic psychrotrophic microorganisms, the surface of PCA agar (OXOID®) received 0.1 mL of dilutions 100, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, using the method of plate dispersion (surface), with incubation of inverted plates at 7 ºC for 10 days. Counts were performed with a colony counter and the results were expressed in CFU/mL (Colony Forming Units per mL). The analysis of total and thermotolerant coliforms was performed using the Multiple Tube Technique, a method recommended by ISO 4831:2006 (ISO, 2006). 1 mL of the sample was inoculated in a series of 3 tubes in Lauryl Sulfate Tryptose Broth (OXOID®) in test tubes containing inverted Durham tubes. A dilution

was performed using saline peptone solution, concentration 10^{-1} and 1 mL was added in a series of 3 tubes Lauryl Sulfate Tryptose Broth (OXOID®). The inoculated tubes will be incubated at 30 ºC for 24 or 48 hours in a bacteriological oven. The tubes with a positive presumptive reaction, evidenced by the production of gas, were then submitted to the confirmatory test in 2% Brilliant Green Lactose Bile Broth (OXOID®). The tubes that showed gas formation in the Brilliant Green Bile 2% test were transferred to Escherichia coli broth (EC) and remained in a water bath for 48 hours at a temperature of 45 ± 0.2 ºC. All microbiological analyzes were performed in triplicates. To verify the quality of the milk, the results found in the analyzes were compared with the limits defined by IN No. 76/2018 of MAPA (Brazil, 2018a), for refrigerated and pasteurized raw milk and by Ordinance No. 370/1997 of MAPA (Brazil, 1997), for sterilized milk. The legislation does not establish amounts of psychrotrophic microorganisms and total and thermotolerant coliforms, however, the results were confronted with such material or with recent scientific publications.

Data Analysis: The data were tabulated using the Excel spreadsheet and statistical tests of Q-square (χ^2) and Shannon's biodiversity index were performed using the Bioestat 5.0 program and principal component analysis (PCA) using the Past program. The genders and the number of microorganisms found in the samples of the three types of milk, raw refrigerated, pasteurized and sterilized, and the two industries analyzed in this study were compared.

RESULTS

The physical-chemical analyzes show that the milk composition parameters: proteins, lactose, fat, EST and ESD and the physical-chemical parameters: temperature, acidity and density are in accordance with the parameters established by legislation, NI No. 76/ 2018 of MAPA (Brazil, 2018a), in industry 2 (Table 1). Industry 1 presented acidity results above the maximum parameter allowed for the three types of milk analyzed (refrigerated, pasteurized and sterilized raw). According to NI No. 76/2018 and Ordinance No. 370/1997 (MAPA), milk acidity levels must remain between 0.14 and 0.18g of lactic acid/100 mL (Brazil, 1997; Brazil, 2018a). In addition, pasteurized milk from industry 1 had a density above the permitted level, with a value of 1.037.

sample having a yellowish color. According to legislation, the SCC analysis and the alizarol test must be performed only on refrigerated raw milk. The average of TBC, mesophilic and psychrotrophic results in refrigerated and pasteurized raw milk are shown in Figure 1. Industry 1 has TBC above the maximum amount allowed by current legislation for refrigerated raw milk, which is up to 900,000 CFU/mL (Brazil, 2018a) in the tanker truck (Figure 1a). The amount of microorganisms in raw milk from industry 1 also extrapolated the maximum value measured by the method, having an estimate of 4,599,000 CFU/mL (4.59 x 10^6 CFU/mL). Industry 2 presented TBC results within the limits of current legislation, and the total bacterial count of refrigerated raw milk from the tanker was 466,000 CFU/mL (4.66 x 10^5 CFU/mL).

Pasteurized milk from industry 1 (Figure 1b) had a mesophilic microorganism count of 9,700 CFU/mL (9.7 x 10^3 CFU/mL). In industry 2, the count of mesophilic microorganisms was 510 CFU/mL (5.1 x 10^2 CFU/mL). NI No. 76/2018 (Brazil, 2018a) does not establish the maximum amounts of mesophilic microorganisms for pasteurized milk. The sterilized milk from both industries did not show colony growth, with the count of mesophilic organisms equal to zero. When evaluating the amount of psychrotrophic microorganisms, industry 1 had a count of 10,000,000 CFU/mL (1.0 x 10^7 CFU/mL) and industry 2 of 6,000,000 CFU/mL (6.0 x 10^6 CFU/mL), for refrigerated raw milk. Pasteurized milk showed counts above 10^3 CFU/mLin both industries, being 80,000 CFU/mL (8.0 x 10^4 CFU/mL) in Industry 1, and 40,000 CFU/mL (4.0 x 10^4 CFU/mL) in industry 2. The count of psychrotrophic microorganisms was much higher than the count of mesophilic microorganisms in the two industries analyzed (Figure 1). The sterilized milk from the industries showed a count of psychrotrophic microorganisms equal to zero. The analysis of total and thermotolerant coliforms showed that the two samples of refrigerated raw milk from the industries of Vale do Taquari - RS presented values equal to 110 MPN/mL, both for total coliforms and for thermotolerant coliforms. Pasteurized milk and sterilized milk from the industries did not present microorganisms of the coliform group in the analyzed samples. Genetic sequencing analyzes showed a total of 51,401 sequences of microorganisms distributed in 41 genera of individuals of the Bacteria Domain. The eight main genera found in the total samples from the industries were: *Bacillus* (14,146), *Kurthia* (9,569), *Streptococcus* (9,222), *Enterobacter* (5,747), *Lysinibacillus* (3,530), *Aeromonas* (1,776),

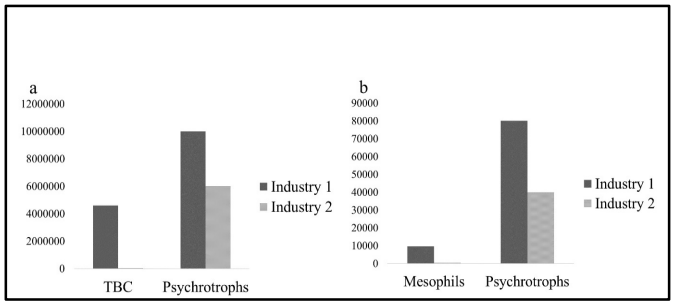
Table 1. Results of physicochemical and compositional analyzes found in milk samples from industries in Vale do Taquari-RS

Parameter		Industry 1				Industry 2			
	Limits	Raw	Past.	Ster.	Average	Raw	Past.	Ster.	Average
Temperature(ºC)	upto 5°C* e 4 °C**	4.8	3.7	22.1	10.2	3.6	4.0	28	11.86
Acidity (g latic acid/100 mL)	0.14 to 0.18	0.67	0.21	0.24	0.37	0.18	0.17	0.18	0.18
Density (g/mL)	1.028 to 1.034	1.028	1.037	1.028	1.031	1.033	1.033	1.033	1.033
Fat (g/100g)	min. 3.00	4.37	3.80	3.00	3.72	3.80	3.20	3.00	3.33
Protein (g/100g)	min. 2.90	3.30	3.24	3.28	3.27	3.27	3.26	3.27	3.27
Lactose (g/100g)	min. 4.30	4.33	4.89	4.72	4.64	4.48	4.92	4.78	4.72
TDE (g/100g)	min. 11.40	13.08	12.42	11.50	12.33	12.59	12.02	11.92	12.17
DDE (g/100g)	min. 8.40	8.71	8.60	8.50	8.60	8.79	8.80	8.90	8.83

Limits and and average of the physical-chemical and composition parameters of raw, pasteurized and sterilized milk from samples from the industries of Vale do Taquari -RS. TDE: Total Dry Extract; DDE: Defatted Dry Extract; *: for refrigerated raw milk; **: for pasteurized milk.

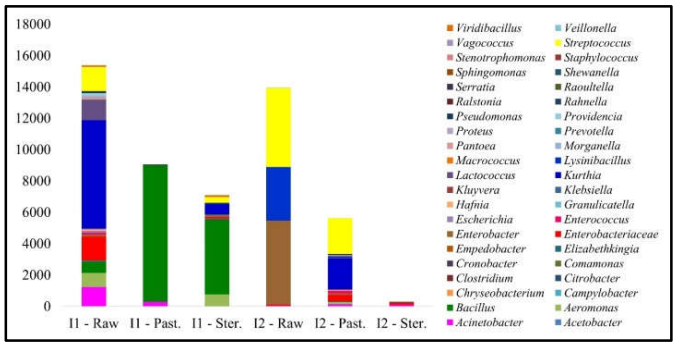
The legislation establishes that the density of milk must be between 1.028 to 1.034 g/mL (Brazil, 2018a). All samples of refrigerated and pasteurized raw milk had a temperature below the maximum allowed at the time of collection, which is up to 5 °C for refrigerated raw milk (Brazil, 2020) and up to 4 °C for pasteurized milk (Brazil, 2020; Brazil, 2018a). The sterilized milk is stored at room temperature and, at the time of collection, it presented a temperature of 22.1 °C in the sample from industry 1 and 28 °C in the sample from industry 2. The SCC analysis showed that the two industries presented values above the maximum allowed by the legislation, which is up to 500,000 SC/mL (Brazil, 2018a), with a value of 1,079,000 SC/mL being obtained in industry 1 and 638,000 SC /mL in industry 2. In industry 1, the amount of SC extrapolated the maximum level obtained by the method, having its value established by estimate. Industry 1 also showed positive alizarol test for refrigerated raw milk, with the

Acinetobacter (1,759) and *Lactococcus* (1,358). Another 33 genera appear with sequences ranging from 263 (*Enterococcus*) to only two (*Clostridium*) and 2,201 sequences were not identified at the genus level, being classified as bacteria of the *Enterobacteriaceae* family. The refrigerated raw milk sample from industry 1 (I1-Raw) presented 15,384 sequences distributed in 32 genera. The 11 main genera were: *Kurthia* (6,907 sequences), *Streptococcus* (1,516), *Lactococcus* (1,307), *Acinetobacter* (1,233), *Aeromonas* (892), *Bacillus* (701), *Providencia* (181), *Enterobacter* (139), *Escherichia* (121), *Enterococcus* (111) and *Hafnia* (101). Another 21 genera appear in the sample with sequences between 1 and 93 (Figure 2). In pasteurized milk (I1-Past.), industry 1 presented a total of 9,052 sequences of microorganisms distributed in 13 genera, the main two being: *Bacillus* (8,699) and *Acinetobacter* (290).



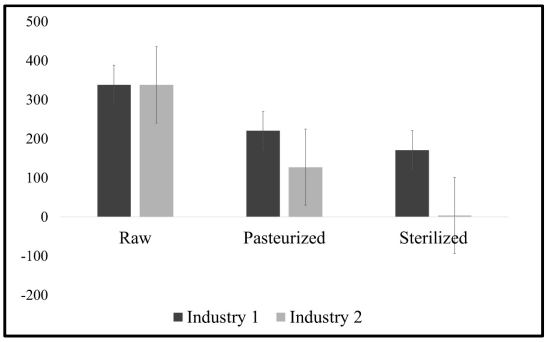
Total bacterial count (TBC) and mesophilic and psychrotrophic microorganisms in CFU/mL, in samples of raw and pasteurized milk from industries in Vale do Taquari. (a) Total bacterial count and amount of psychrotrophic microorganisms in samples of refrigerated raw milk from industries 1 and 2. (b) Count of mesophilic microorganisms and psychrotrophic microorganisms in samples of pasteurized milk from industries 1 and 2.

Figure 1. Average of total bacterial counts, mesophiles and psychrotrophs in samples milk collected at industries in Vale do Taquari- RS



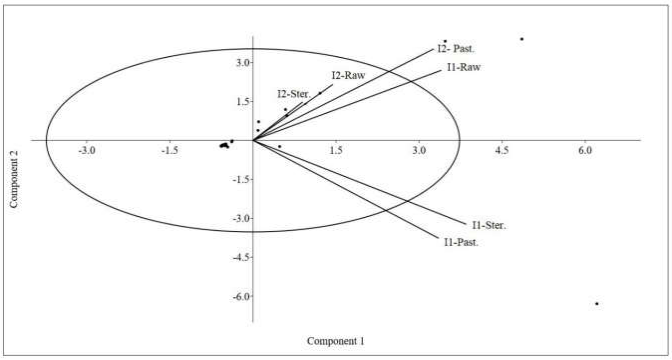
Genus and *Enterobacteriaceae* family found in the milk samples from the industries of Vale do Taquari – RS. I1-Raw: refrigerated raw milk from industry 1; I1-Past.: Pasteurized milk from industry 1; I1-Ster.: Sterilized milk from industry 1; I2-Raw: refrigerated raw milk from industry 2; I2-Past.: Pasteurized milk from industry 2; I2-Ster.: Sterilized milk from industry 2.

Figure 2. Genus and family of microorganisms identified in milk samples from industries in Vale do Taquari - RS



Average amount of microorganisms found in samples of raw, pasteurized and sterilized milk from industries in Vale do Taquari -RS and standard error.

Figure 3. Arithmetic average of the genus found in the milk samples from the industries of Vale do Taquari – RS



Sorting chart using multivariate analysis in the Past program. I1-Raw: refrigerated raw milk from industry 1; I1-Past.: Pasteurized milk from industry 1; I1-Ster.: Sterilized milk from industry 1; I2-Raw: refrigerated raw milk from industry 2; I2-Past.: Pasteurized milk from industry 2; I2-Ster.: Sterilized milk from industry 2.

Figure 4. Perceptual map of multivariate analysis of milk samples collected in industries in Vale do Taquari – RS

In third place, the genus *Pseudomonas* was observed, with only 26 sequences. Another ten genera appear in the sample with less than 10 sequences. The sterilized milk from industry 1 (I1-Ster.) presented a total of 7,090 sequences distributed in 22 genera, nine of which were the main ones: *Bacillus* (4,743), *Aeromonas* (754), *Kurthia* (661), *Streptococcus* (369), *Enterobacter* (145), *Viridibacillus* (113), *Citrobacter* (59) and *Pseudomonas* and *Comamonas* (47). Another 13 genera appear in the sample with less than 21 sequences. The refrigerated raw milk sample from industry 2 (I2-Raw) presented a total of 13,950 sequences, distributed in 11 genera, three of which were the most abundant: *Enterobacter* with 5,351 sequences, *Streptococcus* with 5,053 and *Lysinibacillus*, with 3,402. In fourth place appears the genus *Acinetobacter*, with only 30 sequences. Another seven genres have an insignificant amount of sequences, ranging from 14 to a single sequence. Pasteurized milk from industry 2 (I2-Past.) presented a total of 5,622 sequences distributed in 34 genera, seven of which were more abundant: *Streptococcus* (2,276), *Kurthia (*1,992), *Enterococcus* (150), *Aeromonas* (127), *Acinetobacter* (113), *Enterobacter* (109) and *Lysinibacillus* (103). Another 11 genera appear with sequences between 10 and 50 and 16 genera had a number of sequences less than 10. The sterilized milk from industry 2 (I2-Ster.) showed 303 sequences distributed in 15 genera, three of which were more expressive: *Acinetobacter* (91), *Lactococcus* (17) and *Pseudomonas* (17) and 140 sequences identified at the family level, *Enterobacteriaceae*. Twelve other genera appear in the sample with less than 10 sequences. Using χ^2 to compare the genera found in the milk samples from the industries of Vale do Taquari, it can be seen that there is a statistically significant difference ($p = 0.0001$).

This difference occurs between samples of refrigerated raw milk, pasteurized milk and sterilized milk from the same industry and between the same types of milk from different industries. When calculating the average of genera of microorganisms found in the milk samples from the industries of Vale do Taquari (Figure 3), it is possible to observe that the two samples of refrigerated raw milk have the same and higher average of microorganisms, these being 338.54 and 338 .12 sequences (Industry 1 and Industry 2, respectively). Then comes the sample of pasteurized milk (220.78) and the sample of sterilized milk (171.02) from industry 1. Pasteurized milk from industry 2 showed an average of 127.56 microorganisms and finally, the sterilized milk from industry 2, showed the lowest average of all samples, 3.98 microorganisms. The standard error of the samples ranged from 212.07 in the sample of pasteurized milk from industry 1 and 2.26 in the sterilized milk from industry 2. The sample with the greatest diversity of genera was pasteurized milk from industry 2 with 34 genera, followed by the raw milk sample from industry 1, with 32 genera, and the sample of sterilized milk also from industry 1, with 22 genera. The raw milk sample from industry 2 had the lowest number of genera with only 11. The sterilized milk sample from industry 2 had 15 genera and the pasteurized milk sample from industry 1 had 13 genera. The Shannon diversity index of the milk samples analyzed in this study ranged from 0.1961 in the pasteurized milk sample from industry 1 to 1.845 in the refrigerated raw milk sample also from industry 1. The sterilized milk sample from industry 2 had an index of 1.649, followed by the pasteurized milk sample from industry 2 (1.54), the sterilized milk sample from industry 1 (1.206) and the refrigerated raw milk sample from industry 2 (1.108). The Shannon-Weaver diversity index considers equal weight between rare and abundant species and the lower the index value, the lower the degree of uncertainty and, therefore, the sample diversity is low (Furtado and Vieira, 2020). The analysis of the principal components (PCA) of the genera found in the samples from the analyzed industries (Figure 4) demonstrates that there is an association between the refrigerated raw milk sample from industry 1 and the three samples from industry 2 (refrigerated raw, pasteurized and sterilized). The sample of pasteurized milk from industry 1 is associated with the sample of sterilized milk from industry 1. Component 1, sample of refrigerated raw milk from industry 1, explains 35.07% of the results and component 2, sample of pasteurized milk from industry 1 explains 31.05% of the results, together these components explain 66.12% of the results.

DISCUSSION

Physico-chemical parameters and milk composition of industries: Industry 1 showed a positive alizarol test for raw milk and acidity above the maximum level allowed for refrigerated, pasteurized and sterilized raw milk. Pasteurized milk from the same industry presented density above the maximum allowed. The alizarol test aims to verify the stability of the milk, confirmed by the formation of a brick color in the analyzed sample. When the milk is unstable, clumps form and its color may be violet or yellow, being rejected by the industries. The yellow color represents acidification of the sample, usually caused by microbial activity and the violet color indicates fraud by addition of constituents, such as acidity reducers, with sodium bicarbonate being the most used (Ulisses *et al*., 2022). The sample from industry 1 showed a yellowish color, indicating acidification of the sample by microbial metabolism, and this same sample showed acidity above the allowed level. According to Sandoval and Ribeiro (2021), milk acidity is characterized by the presence of microorganisms that metabolize lactose, forming lactic acid. The fact that pasteurized milk and sterilized milk from industry 1 (0.21 and 0.24 g lactic acid/100 mL, respectively) present milk acidification may be a result of acidity much higher than that allowed in refrigerated raw milk (0 .67 g lactic acid/100 mL), which was almost four times higher than the maximum level allowed by legislation, which is up to 0.18 g lactic acid/100 mL. In addition, pasteurized and sterilized milk showed a higher number of sequences of microorganisms than those observed in industry 2, with this difference being more expressive in sterilized milk (7,090 sequences: industry 1 and 303 sequences: industry 2). The density of milk is variable, depending on its composition and is used to control fraud, up to a certain limit, the main ones being previous skimming and the addition of water. Samples with densities below or above that determined by NI No. 76 (Brazil, 2018a) cause the rejection of milk by industries (Ulisses *et al*., 2022). The pasteurized milk sample from industry 1 showed higher density than recommended. According to Souza *et al*. (2018), density above the established levels may indicate that the milk was skimmed or that some corrective product was added.

Microbiological parameters of industrial milk: Microbiological analyzes show that industry 1 has SCC and TBC above the levels allowed by current legislation for refrigerated raw milk. Industry 2 presented TBC within the established, but had SCC levels above the limits established by legislation. The number of somatic cells is 69% higher in Industry 1 compared to Industry 2 (441,000 SC/mL more) and the TBC is 10 times higher in Industry 1 compared to Industry 2. Chemical composition and microbiological quality of milk are extremely important in the production of milk and dairy products. In this context, SCC and TBC have a great influence on the organoleptic characteristics, as well as the durability and shelf life of the milk (Martins Junior *et al*., 2021). There is a direct relationship between SCC and milk quality. This parameter is a well-established and commonly used milk quality criterion for evaluating the intramammary health status of both individual animals and bulk milk tanks. Udder infection is considered the most frequent cause of increased SCC in bovine milk and is mainly caused by pathogenic microorganisms. In the milk of uninfected animals, epithelial cells form about 50% of somatic cells, with the remainder derived from blood and leukocytes. Polymorphonuclear leukocytes, macrophages and lymphocytes represent approximately 25%, 15% and 10%, respectively, of SCC (Moradi *et al*., 2020). High amounts of SCC, in addition to reflecting on the quality of milk, closely interfere with the industrial yield of dairy products (Martins Junior *et al*., 2021). TBC is another important tool in monitoring the quality of raw milk, being an indicator of hygienic-sanitary conditions in obtaining milk. This count is often directly correlated with the count of psychrotrophic bacteria (PBC) in the product (Lampugnani *et al*., 2018). Milk collected shortly after milking often has a low TBC and this may be due to the milk not yet having come into contact with biological contaminants. A very high bacterial count can be caused by the absence or poor hygiene at the time of milking (Melo *et al*., 2021). For Hahne *et al*. (2019), the microbiota of bulk tank milk with high bacterial counts is predominated by cold-adapted species, such as psychrotrophic microorganisms, which have high rates of microbial growth at low temperatures. Industry 1 high TBC is in line with high sample acidity and positive alizarol test. For Fagnani *et al*. (2016), the relationship between TBC and acidity has been widely studied in milk and the main cause of acidity comes from the metabolism of mesophilic aerobic microorganisms. Thus, both acidity, high TBC and the alizarol test indicate microbial activity in refrigerated raw milk from industry 1, extending to processed milks. The counting of mesophilic microorganisms can be performed in pasteurized and sterilized milk. According to MAPA Ordinance No. 370/1997, the number of mesophiles must not exceed 100 CFU/mL in UHT milk (Brazil, 1997). Industry 1 showed a greater number of colonies of mesophilic microorganisms (9,700 CFU/mL) compared to Industry 2, which was only 510 CFU/mL, for pasteurized milk. Industry 1 had a number of mesophiles 19 times greater than industry 2, which is in agreement with the TBC in refrigerated raw milk, which was also much higher in industry 1.

When evaluating the amount of psychrotrophic microorganisms in refrigerated raw milk, industry 1 had a higher count (10,000,000 CFU/mL) than industry 2 (6,000,000 CFU/mL). The same occurs for pasteurized milk, with the count being 80,000 CFU/mL, in industry 1, and 40,000 CFU/mL, in industry 2. Psychrotrophic microorganism is a general term for a class of microorganisms that are able to grow at low temperatures. These microorganisms have the ability to produce enzymes, which can impair the quality of milk and dairy products (Wei *et al*., 2019). The proteolytic and lipolytic enzymes produced by these microorganisms are associated with technological and sensory changes in the product, even after processing, due to their heat resistance capacity (Lampugnani *et al*., 2018). The count of psychrotrophic bacteria (PBC) is considered an important indicator that determines the quality of raw milk and final dairy products. The CBP generally required to initiate spoilage in milk is about 10^6 CFU/mL (Yang *et al*., 2020). In most countries, raw milk is not processed immediately after milking and is therefore kept refrigerated until processing. The entire process can take up to 5 days, depending on milk collection intervals and transport distances, which results in increased numbers of psychrotrophic microorganisms. Furthermore, prolonged cold storage of raw milk can influence the microbial diversity of that milk (Zhang *et al*., 2020). The samples of refrigerated raw milk from the analyzed industries showed psychrotrophic counts up to 10^7 CFU/mL (industry 1) and 10^6 CFU/mL (industry 2), evidencing levels of microorganisms prone to milk deterioration and alteration of its characteristics organoleptic.

The two industries evaluated showed a higher number of psychrotrophic microorganisms than the number of mesophilic microorganisms (pasteurized milk) and the total bacterial count (refrigerated raw milk). In refrigerated raw milk, the psychrotrophic count was twice as high as the TBC for industry 1 (10,000,000 CFU/mL and 4,599,000 CFU/mL, respectively) and more than 10 times higher for industry 2 (6,000 .000 CFU/mL and 466,000 CFU/mL, respectively). The fact that industry 1 has a TBC above that established by legislation explains the smaller difference between mesophiles and psychrotrophs in relation to industry 2. In pasteurized milk, the count of psychrotrophic microorganisms was 8 times higher than the count of mesophilic microorganisms in industry 1 (80,000 CFU/mL and 9,700 CFU/mL, respectively) and 80 times higher in industry 2 (40,000 CFU/mL and 510 CFU/mL, respectively). The average TBC of refrigerated raw milk samples was 2,532,500 CFU/mL, that of mesophilic samples from pasteurized milk was 5,105 CFU/mL and that of psychrotrophs was 8,000,000 CFU/mL for raw milk and 60,000 CFU/mL for pasteurized milk. Although there is no established maximum level, in good quality milk, the psychrotrophic count should be no more than 10% of the total mesophilic aerobic or TBC count. In heavily contaminated milk, the count of psychrotrophs increases proportionally, and can be much higher than the number of mesophiles (Mariotto *et al*., 2020). Prolonged refrigeration may have been responsible for such a large difference between the amount of mesophilic and psychrotrophic

microorganisms in the samples from the industries analyzed in this study. The analysis of total and thermotolerant coliforms showed the presence of the two groups of microorganisms in the refrigerated raw milk of both analyzed industries, in an amount of 100 MPN/mL. Coliforms are defined as aerobic or facultative anaerobic, gram-negative, non-spore forming rods capable of fermenting lactose, resulting in gas and acid production at 35 °C in 48 h (Godziszewska *et al*., 2018). Coliforms have been used in the dairy industry since the early 20th century to identify milk processed under unsanitary conditions or where contamination has occurred after pasteurization. This group is formed by members of the Enterobacteriaceae family and, historically, has shown an important role in the deterioration of fluid milk. Despite the advantages of their use as indicators, current research indicates that coliforms are decreasing considerably in fluid milk (Alles *et al*., 2018). Coliform microorganisms are represented by four main genera: Escherichia, Klebsiella, Citrobacter and Enterobacter (Godziszewska *et al*., 2018). Genetic sequencing demonstrated the presence of the four genera of coliforms in the refrigerated raw milk samples analyzed in this study.

Although the samples of pasteurized milk and sterilized milk did not show total and thermotolerant coliforms in the microbiological analyses, the sequencing showed the presence of the genera Enterobacter, Citrobacter and Escherichia in the pasteurized milk of industry 2 and Enterobacter, Citrobacter, Klebsiella in the sterilized milk of the industry 1. Godziszewska *et al*. (2018), in their study, detected microorganisms from the coliform group in 95% of bulk tank milk samples. For Masiello *et al*. (2019), coliforms are often isolated from raw milk and pasteurized milk, and their presence in processed milk can be explained by contamination after processing. For Odenthal *et al*. (2016), heat treatment of milk by UHT is efficient in inactivating members of the Enterobacteriaceae family, but in this study, genera of this family were found in sterilized milk. Genetic sequencing is a more efficient tool that performs a thorough analysis of the sample, which would explain why the coliform group was not detected by the traditional microbiological method. This is also demonstrated in the analysis of mesophilic and psychrotrophic microorganisms in sterilized milk, which did not show the growth of colonies in the microbiological method, however, in the sequencing, sequences of microorganisms were found in both industries, being very expressive in the case of industry 1 (7,090) and less expressive in industry 2 (303). Industry 1 showed higher amounts for most of the microbiological parameters evaluated in this study. Compared to industry 2, industry 1 has a higher TBC, a greater number of mesophiles, psychrotrophs, somatic cells, in addition to a greater amount of microorganism sequences in the three types of milk analyzed, raw refrigerated, pasteurized and sterilized. Despite this, both industries show a reduction in the amount of mesophilic and psychrotrophic microorganisms and in the amount of sequence of microorganisms from refrigerated raw milk to processed milk (pasteurized and sterilized). In industry 1, the number of mesophiles reduced from 4,599,000 CFU/mL in refrigerated raw milk to 9,700 CFU/mL in pasteurized milk and zero in sterilized milk. The number of psychrotrophs reduced from 10,000,000 CFU/mL in refrigerated raw milk to 80,000 CFU/mL in pasteurized milk and zero in sterilized milk. The number of microorganism sequences reduced from 15,384 in refrigerated raw milk to 9,052 in pasteurized milk (41.15%) and 7,090 in sterilized milk (53.91%). In industry 2, the number of mesophiles reduced from 466,000 CFU/mL in refrigerated raw milk to 510 CFU/mL in pasteurized milk and zero for sterilized milk. The number of psychrotrophs reduced from 6,000,000 CFU/mL in refrigerated raw milk to 40,000 CFU/mL in pasteurized milk and zero in sterilized milk. The number of microorganism sequences reduced from 13,950 in refrigerated raw milk to 5,622 in pasteurized milk (59.69%) and 303 in sterilized milk (2.17%). This indicates that the beneficiation processes of industries have been efficient in reducing the amount of microorganisms.

Industry milk microbiome: When observing the total of the genera found in the six analyzed samples, eight genera are considered more abundant. The genus Bacillus (14,146) represents 27.52% of the total sample, the other 7 (*Kurthia, Streptococcus, Enterobacter, Lysinibacillus, Aeromonas*, *Acinetobacter* and *Lactococcus*) represent 64.12% of the total. The refrigerated raw milk sample from industry 1 (I1-Raw) presented as the most abundant genera: *Kurthia, Streptococcus, Lactococcus, Acinetobacter, Aeromonas, Bacillus, Providencia, Enterobacter, Escherichia, Enterococcus* and *Hafnia*. The genus *Kurthia*alone represents 44.89% of the amount of sequences present in the sample, and the other 10 together represent 40.96%. *Kurthia* is a genus of proteolytic and lipolytic psychrotrophic microorganisms from Brazilian refrigerated raw milk. This genus also has some mesophilic microorganisms (Ribeiro Junior *et al*., 2019). Hahne *et al*. (2019), in their study, found species of the genus Streptococcus as dominant in the microbiota of raw milk samples from bulk tanks. Recent research on the prevalence of mastitis pathogens has reported that Streptococcus is the most common type of pathogen associated with clinical mastitis (Smith *et al*., 2020). Lactococcus is the most extensively studied genus of lactic acid bacteria (LAB) as these bacteria are extensively used in the food industry (Guo *et al*., 2019). Species of the genus Lactococcus were recently associated with the occurrence of clinical mastitis. This may be related to changes in the environment, which facilitate their growth and introduction into the udder, or to biochemical improvements and advanced molecular techniques that have allowed the accurate identification of Lactococcus spp. instead of misclassification in Streptococcus spp. (Smith *et al*., 2020). For Mallappa *et al*. (2020), *Lactococcus*, together with *Lactobacillus, Leuconostoc,Streptococcus* and *Enterococcus* are the most common LAB genera in milk. In addition to these, psychrotrophic microorganisms, yeasts and molds, which establish themselves particularly during cold storage, are the main components of dairy products. Acinetobacter is a genus of psychrotrophic species also very common in raw milk. This genus is ubiquitous in the environment and milk contamination can result from the place where the animals are, such as the stable, hay, air or from ineffective cleaning processes (Hahne *et al*., 2019).

The fourth most abundant genus in the sample was Aeromonas, composed of emerging pathogens capable of colonizing and infecting several hosts. This genus can be isolated from foods such as vegetables, beef and pork. In humans, these microorganisms are capable of causing infections of the gastrointestinal system (Pessoa *et al*., 2019). Aeromonas is one of the main lipase-producing genera, along with Pseudomonas, Moraxella, Acinetobacter, Achromabacter, Aeromonas, Serratia and Alcaligenes. Lipases produced by psychrotrophic microorganisms in raw milk can withstand the heat treatment used in the dairy industry, causing flavor defects in manufactured products that have a long shelf life, such as UHT milk (Deeth, 2021). Bacillus is also one of the genera commonly found in milk and dairy products. Some species of this genus have the potential for biofilm formation, persisting in the industrial environment (Lindsay *et al*., 2022). Spores of some species of *Paenibacillus* (formerly classified as Bacillus) can survive heat treatment in raw milk and can withstand temperatures up to 130 °C. In addition, enzymes produced within biofilms degrade the protein and lipid components of milk, altering its sensory and nutritional properties (Elegbeleye & Buys, 2022). *Providencia* are urease-producing gram-negative microorganisms belonging to the Enterobacteriaceae family. Although these species are present as normal flora of the human intestinal tract, they are opportunistic pathogens, especially in immunocompromised people. Animals such as cattle, sheep, insects, worms, cats, birds, dogs and reptiles are reservoirs of Providencia, in addition to being present in water. Raw milk is a potential source of microorganisms of this genus, and contamination can occur during the milking process, through animal feces or can be related to subclinical mastitis (Al-Gburi, 2020). Like *Providencia*, the *Enterobacter* and *Escherichia* genera are members of the *Enterobacteriaceae* family. The *Enterobacter* genus is ubiquitous in nature and is widely dispersed in various ecosystems and niches such as water, soil, plants, faeces and skin, as well as the alimentary tract of humans, but it has also been isolated in milk (Khalifa, 2020). According to Ionnaou (2019), in recent decades several members of Enterobacteriaceae have been reclassified as unique species within the genus *Escherichia: Escherichia vulneris, Escherichia blattae, Escherichia fegusonii* and *Escherichia*

hermannii. Some of these species can cause infections in humans, as is the case with *E. hermannii*and *Escherichia coli. Enterococcus* are part of the microbiota of many raw and pasteurized foods. These microorganisms have a dual nature, with useful and harmful microorganisms. Scientific evidence confirms the discovery of strains with probiotic and functional potential. Species such as *Enterococcus faecalis* and *Enterococcus faecium* are used as probiotics for humans and also as veterinary food supplements (Giraffa, 2022). *Hafnia* are gram-negative, rod-shaped microorganisms belonging to the *Enterobacteriaceae* family, which has opportunistic pathogenic species of humans and animals. This genus is common in foods and has often been isolated from spoiled food products, especially in raw protein foods stored under refrigeration, such as fish, meat, and milk. This genus can form biofilms, which adhere to the solid surface, which is a potentially important factor that causes food contamination and spoilage (Zhu *et al*., 2019).

According to Olajide and LaPointe (2022), the diversity of microorganisms in raw milk can come from the animal, milking equipment, transport, storage or the environment. Microorganisms in milk can be harmful (pathogenic, spoilage) or beneficial. Some microorganisms, such as LAB, can be used to produce fermented dairy foods when grown under controlled conditions. Milk is a good medium for microorganisms to grow, so controls over storage temperature and hygiene during production and processing are essential to maintain an acceptable product. According to Yang *et al*. (2020), the most abundant psychrotrophic genera in raw milk are *Pseudomonas, Acinetobater, Flavobacterium, Sphingobacterium* and *Serratia,* for gram-negatives, and *Lactococcus, Aerococcus, Bacillus, Kurtha* and *Staphylococcus* for gram-positives. In their study, three genera were reported with high frequency: *Pseudomonas,Lactococcus* and *Acinetobacter.* Zhang *et al*. (2019), reports as the main genera of psychrotrophs found in raw milk with gram-negative properties: *Pseudomonas, Aeromonas, Serratia, Acinetobacter, Alcaligenes, Achromobacter, Enterobacter* and *Flavobacterium* and gram-positive: *Bacillus, Clostridium, Corynebacterium, Microbacterium, Micrococcus, Arthbacter, Staphylococcus* and *Carnobacterium.* In this study, the genera *Aeromonas, Bacillus, Providencia, Acinetobacter, Enterobacter, Pseudomonas* and *Kurthia* were found, as well as *Viridibacillus.* Pasteurized milk from industry 1 (I1-Past.) presented only three abundant genera: *Bacillus, Acinetobacter* and *Pseudomonas*, and the genus *Bacillus*, with 8,699 sequences, represents 96.10% of the total sample (9,052). It is well understood that pasteurization of milk and dairy products keeps consumers safe from foodborne illness, while failure to heat treatment can result in foodborne illness outbreaks (Lindsay *et al*., 2021). For Wei *et al*. (2019), the quality of raw milk is important to determine the quality of processed milk and industrialized dairy products. The genus *Pseudomonas*, with only 26 sequences, represents 0.28% of the total sample and is well known for its ability to produce biofilms. Biofilm formation of microorganisms in the storage tank leads to increased contamination of milk because biofilm-associated organisms exhibit high levels of resistance to cleaning and disinfection (Hanhe *et al*, 2019).

Species of nine main genera were found in the sterilized milk from industry 1 (I1-Ster.): *Bacillus, Aeromonas, Kurthia, Streptococcus, Enterobacter, Viridibacillus*, *Citrobacter*, *Pseudomonas* and *Comamonas*.The genus *Bacillus* (4,743) is the most abundant and represents 66.89% of the sequences present in the sample (7,090). *Viridibacillus*represents 1.59% (113 sequences) of the total microorganisms present in the sample. This genus is composed of microorganisms that are ubiquitous in nature and have already been isolated throughout the dairy chain. In addition, members of this genus are capable of producing spores that survive in adverse conditions. Thus, the ability to reduce the presence or control the growth of psychrotolerant spore formers in the dairy system has the potential to considerably improve the quality of fluid milk (Buehler *et al*., 2018). For Alles *et al*. (2018), spore-forming gram-positive bacteria represented the majority of milk bacteria and their main groups are the *Bacillales*family, with the genera *Bacillus* and *Viridibacillus*being the most frequent. The genus *Citrobacter* (with 59 sequences) represents 0.83% of the total sample. This genus is composed of gram-negative, aerobic and facultative anaerobic bacteria belonging to the *Enterobacteriaceae* family, commonly disseminated in nature. Recent studies have shown Citrobacter infections in fish and the indiscriminate use of antibiotics has given rise to resistant species (Royam; Nachimuthu, 2020). This genus has been found in fruits and vegetables, due to its proximity to the soil and inadequate handling (Adegun *et al*., 2019). Despite the low incidence in the sample, its presence in UHT milk is a cause for concern, and may be an indication of post-processing contamination. The genus *Comamonas*, as well as *Pseudomonas*, is not very expressive in the sample. This genus is formed by gram-negative, non-fermentative and rod-shaped bacteria, most of which are aerobic chemoheterotrophs considered non-pathogenic to humans (Wu *et al*., 2018). The refrigerated raw milk sample from industry 2 (I2-Raw) has only three main genera: *Enterobacter, Streptococcus* and *Lysinibacillus,* representing 38.35%, 36.22% and 24.38% of the total sample (13,950 sequences), respectively.

Together, these three genres add up to 13,806 sequences, that is, 98.95% of the total sample. Members of the genus *Lysinibacillus*have been isolated from diverse environments and are reported to be potential symbionts of animals and plants, as well as free-living soil microorganisms. These microorganisms have long been known as insect biocontrol agents and are also important plant growth-promoting bacteria (Hashmi *et al*., 2020). In the pasteurized milk from industry 2 (I2-Past.) seven main genera were found: *Streptococcus, Kurthia, Enterococcus, Aeromonas, Acinetobacter, Enterobacter* and *Lysinibacillus.* The two main genera, *Streptococcus* and *Kurthia* together represent 75.91% of the total sample (5,622 sequences). Ding *et al*. (2020) found as main genera in pasteurized milk: *Pseudomonas, Corynebacterium, Streptococcus, Cyanobacteria.* Pasteurization can eliminate some pathogenic microorganisms, but there are species capable of withstanding heat treatment. The clear identification of the microorganisms present in the samples is important for the rigorous control of pasteurization in industries. The genera *Streptococcus* and *Pseudomonas* were found in pasteurized milk from industries 1 and 2, in agreement with the study by Ding *et al*. (2020). An important situation is post-pasteurization contamination (PPC) that can occur due to poor hygiene practices in the industry or from existing biofilms on processing equipment (Elegbeleye; Buys, 2022). Post pasteurization contamination is still an obstacle for some industries.

Studies suggest that 40 to 50% of conventional pasteurized fluid milk shows evidence of post-process contamination. CPP is associated with rapid bacterial growth producing unacceptable sensory characteristics, which often lead to spoilage before the product's shelf life (Alles *et al*., 2018). In the sterilized milk from industry 2 (I2-Ster.) three main genera were found, *Acinetobacter, Lactococcus* and *Pseudomonas.* The genus *Acinetobacter* represents 30.03% of the total sample. *Lactococcus* and *Pseudomonas* represent 5.61% each. In this sample, 140 sequences of microorganisms of the *Enterobacteriaceae* family, not identified at the genus level, were identified, which represents 46.20% of the sample. As they belong to the *Enterobacteriaceae* family, they should not be present in UHT milk, indicating possible post-processing contamination. In the present study, the genus *Bacillus* was the most abundant in the total of samples. This genus is commonly found in milk and dairy products and has the potential to form biofilms and spores resistant to heat treatment, which explains its presence in processed milk samples. The refrigerated raw milk samples showed to have in common psychrotrophic, mastitogenic and intestinal origin genera, *Acinetobacter, Enterobacter* and *Streptococcus*. Pasteurized milk samples showed the Acinetobacter and Pseudomonas genera in common, both of which are formed by psychrotrophic microorganisms. The sterilized milk samples showed *Streptococcus, Enterobacter, Kurthia* and *Pseudomonas*. *Kurthia* and *Pseudomonas* are psychrotrophic genera and *Enterobacter* and *Escherichia* are associated with contamination of fecal origin. Improved access to genome-based culture-independent methods has generated great interest in defining the bovine milk microbiome. Several bacterial

genera are routinely identified from milk samples, but the origin and function of these organisms are uncertain, and environmental factors have been shown to strongly influence the composition of these bacterial populations, as sources of microbial DNA may include bacteria introduced from the skin or environment. Understanding the bovine milk microbiome has been hampered by the lack of standardized methods used to collect, process and evaluate bovine milk samples. Furthermore, contamination of samples with bacterial DNA from laboratory reagents is a well-known problem that has affected the results of studies with bovine milk samples (Ruegg, 2022).

CONCLUSION

The analysis of samples of refrigerated, pasteurized and sterilized raw milk from the dairy industries of Vale do Taquari showed that industry 1 presented acidity above the maximum allowed for the three types of milk, TBC and alizarol positive for refrigerated raw milk and density outside the level set for pasteurized milk. The two industries presented SCC above the limit established by the current legislation and obtained the same number of microorganisms from the group of total and thermotolerant coliforms for refrigerated raw milk. The count of psychrotrophic microorganisms was above the recommended and was superior to the TBC and the count of mesophilic microorganisms, in refrigerated raw milk and in pasteurized milk from both industries. The number of SCC, mesophiles and psychrotrophs was higher in industry 1. The samples showed great diversity of genera. The main microorganisms found were psychrotolerant, such as *Kurthia, Acinetobacter, Viridibacillus*, biofilm formers, such as *Pseudomonas, Bacillus,*mastogenic such as *Streptococcus* and lactic acid such as *Lactococcus,* in the three types of milk analyzed. In addition, genera of microorganisms considered harmful such as *Escherichia, Citrobacter, Aeromonas* and *Enterobacter* were found even in processed milk. The physical-chemical, compositional and microbiological analyzes of milk produce an efficient assessment of its quality. However, these analyzes can be used in conjunction with high-throughput sequencing, providing a complete diagnosis, precisely identifying the microorganisms present in the samples, thus enabling the tracking of possible failures or the improvement of the production process throughout the dairy chain.

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SCIENTIFIC ARTICLE
Food Microbiology

Microbiological profile of raw refrigerated and processed bovine milk at dairy industries from Vale do Taquari, Rio Grande do Sul, Brazil

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ABSTRACT

Milk is an essential food, widely consumed by the population. Brazil is one of the world's largest producers of milk. Milk quality is influenced by several factors in all its stages of production. The aim of this study was to determine the microbiological profile of refrigerated and processed raw bovine milk from industries in Vale do Taquari, state of Rio Grande do Sul, Brazil, using metagenomic analysis. A total of six samples were collected, one of refrigerated raw milk from the tanker truck, one of pasteurized milk and one of milk sterilized by the ultra-high temperature (UHT) process, in each of the industries. The identification of the milk microbiota was performed by sequencing the 16S rRNA gene. The results show that refrigerated raw milk has a greater number of microorganisms, followed by pasteurized milk and sterilized milk, successively. Processed milk showed the presence of beneficial microorganisms such as *Streptococcus thermophilus* and *Streptococcus macedonicus*. Nevertheless, even UHT milk showed the presence of microorganisms considered harmful, such as the *Bacillus cereus* group, *Aeromonas dhakensis*, *Enterobacter bacterium* and *Acinetobacter haemolyticus*. Metagenomics is a valuable tool for the thorough evaluation of the milk microbiota in order to implement the processing stages in industries.

Keywords: microorganisms; microbiome; genetic sequencing.

INTRODUCTION

Brazil is considered the fifth largest world milk producer and obtained a production of 6,555,592 thousand liters in the first quarter of 2021, with Rio Grande do Sul accounting for 840,063 thousand liters (IBGE, 2021). The Vale do Taquari region is responsible for much of the state production and this activity is the basis of the economy in the small municipalities that compose it.

Milk is a rich food that has nutritional characteristics essential to humans. The refrigerated raw milk must remain at a maximum temperature of 5 °C until it reaches the industry (BRAZIL, 2018a) and to be consumed, it needs to go through processing stages. This processing can occur in two ways: pasteurization or ultra-high temperature (UHT) sterilization. The Ministry of Agriculture, Livestock and Supply's Normative Instruction No. 76 provides information on the identity and quality characteristics that the refrigerated raw milk, pasteurized milk and pasteurized milk type A should present (BRAZIL, 2018b), and Ordinance No. 370 regulates the characteristics that UHT milk must present (BRAZIL, 1997).

Milk microbiota can be composed of microorganisms beneficial to human health or even deteriorating or pathogenic microorganisms. Bovine milk microbiota has been intensively used with the objective of evaluating and improving animal

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health and ensuring milk quality, additionally to its consumption safety. High-throughput sequencing has been extensively used in determining the microbial community in milk and dairy products, with the objective of identifying microorganisms present that may be difficult to cultivate or are in low concentration (RUSSO et al., 2020).

High-throughput sequencing is used to perform metagenomics, a research field which main objective is to verify the total content of microorganisms present in the analyzed sample (SUDARIKOV et al., 2017). The use of metagenomics can be of great value in the food industry, as it allows the identification of microbiological amplitude, with possible applications in the improvement of products or identification of failures in the processing stages (YAP et al., 2020).

The aim of this study was to determine the microbiological profile of refrigerated and processed raw bovine milk from industries in Vale do Taquari, Rio Grande do Sul, using metagenomic analysis. Refrigerated raw milk of tanker trucks, pasteurized milk and milk sterilized by the UHT process of two industries in the region were tested. The characterization of the milk microbiota was performed by sequencing the 16S rRNA gene, which allows the identification of multiple microorganisms.

MATERIAL AND METHODS

The study was conducted in the Vale do Taquari region, Rio Grande do Sul, Brazil. The sampling and analyses occurred in March and April of the current year. A total of six samples were collected in two dairy industries in two municipalities, one of refrigerated raw milk from the tanker truck, one of pasteurized milk and one of milk sterilized by the UHT process, in each of the industries. The companies received the name C1 (company 1) and C2 (company 2) and the milk types received their initial, being "R" for refrigerated raw milk, "P" for pasteurized milk, and "S" for sterilized milk. At the time of sampling, the sample temperature was measured using an Incoterm thermometer (model 5135). The samples were collected with 100-mL sterilized plastic bottles and packed in a polystyrene box with ice, which kept the temperature of the samples below 12 °C.

Bacteria identification was performed using high-throughput sequencing of the 16S rRNA gene V3/V4 regions. Amplification with primers for region V3-V4 of the rRNA 16S, 341F (CCTACGGGRSGCAGCAG), and 806R (GGACTACHVGGGTWTCTAAT) gene was performed. polymerase chain reaction reactions were performed in triplicates, with the following conditions: 95 °C for 5 min, 25 cycles of 95 °C for 45 s, 55 °C for 30 s and 72 °C to 45 s and a final extension of 72 °C for 2 min.

Genomic libraries were sequenced using the MiSeq Sequencing System. For single-end sequencing, the V2 kit with 300 cycles was used. The sequences were analyzed through the Sentinel pipeline. In the Sentinel pipeline fastq files were evaluated for Phred quality using the FastQC v.0.11.8 software. Therefore, fastq files were subjected to the trimming of primers and sequences with low quality (Phred < 20). The software used for this purpose was built in Python v.3.6, this being inspired by the features of the BioPython project. For paired-end data, before the trimming step, two pairs of files (R1 and R2) were joined in a single file using pandaseq v.2.11. Clusters with abundance smaller than two were removed from the analyses, as such structures are usually related to chimera sequences. Taxonomic identifications were performed with BLASTn v.2.6.0, using a proprietary or public database as reference. As for the definition of a species, among the 20 hits returned for each cluster, a Python instruction evaluated whether one of the three items would be met by the hits: (i) higher bit-score; (ii) lower value; and (iii) greater representation taxonomies. The data were tabulated using excel and biostatistical analyses were performed using the Past software.

RESULTS AND DISCUSSION

The analysis of milk samples collected in the industries of Vale do Taquari (RS) confirmed the presence of 51,401 sequences from a single kingdom, Bacteria. Three phyla (Bacteroidetes, Firmicutes and Proteobacteria), nine classes (Alphaproteobacteria, Bacilli, Bacteroidia, Betaproteobacteria, Clostridia, Epsilonproteobacteria, Flavobacteriia, Gammaproteobacteria and Negativicutes), 15 orders, 21 families, and 41 genera were found (Table 1). RYU et al. (2021), in their study with refrigerated raw milk, reported having found microbiota with the prevalence of the phyla Proteobacteria, Bacteroidetes and Firmicutes, Actinobacteria—three in common with this study.

Table 1. Orders, families and genera found in samples of refrigerated, pasteurized and sterilized raw milk from the companies of Vale do Taquari, Rio Grande do Sul, Brazil.

Orders	Families	Genera	
Aeromonadales	*Acetobacteraceae*	*Acetobacter*	*Lysinibacillus*
Alteromonadales	*Aeromonadaceae*	*Acinetobacter*	*Macrococcus*
Bacillales	*Bacillaceae*	*Aeromonas*	*Morganella*
Bacteroidales	*Burkholderiaceae*	*Bacillus*	*Pantoea*
Burkholderiales	*Campylobacteraceae*	*Campylobacter*	*Prevotella*
Campylobacterales	*Carnobacteriaceae*	*Chryseobacterium*	*Proteus*
Clostridiales	*Clostridiaceae*	*Citrobacter*	*Providencia*
Enterobacteriales	*Comamonadaceae*	*Clostridium*	*Pseudomonas*
Flavobacteriales	*Enterobacteriaceae*	*Comamonas*	*Rahnella*
Lactobacillales	*Enterococcaceae*	*Cronobacter*	*Ralstonia*
Pseudomonadales	*Erwiniaceae*	*Elizabethkingia*	*Raoultella*
Rhodospirillales	*Flavobacteriaceae*	*Empedobacter*	*Serratia*
Selenomonadales	*Moraxellaceae*	*Enterobacter*	*Shewanella*
Sphingomonadales	*Planococcaceae*	*Enterococcus*	*Sphingomonas*
Xanthomonadales	*Prevotellaceae*	*Escherichia*	*Staphylococcus*
	Pseudomonadaceae	*Granulicatella*	*Stenotrophomonas*
	Shewanellaceae	*Hafnia*	*Streptococcus*
	Sphingomonadaceae	*Klebsiella*	*Vagococcus*
	Staphylococcaceae	*Kluyvera*	*Veillonella*
	Streptococcaceae	*Kurthia*	*Viridibacillus*
	Veillonellaceae	*Lactococcus*	
	Xanthomonadaceae		

The analyses also demonstrated the presence of 87 species (Table 2). Of the total species, 59% (52 species) had abundance smaller than 50 sequences, that is, less than 1% of the total individuals found (51,401). For TAPONEN et al. (2019), microorganisms appearing in small quantities should not be considered as they may indicate sporadic species. In addition, the microbiota of bovine milk is an extremely complex issue, as the results obtained for bovine milk using the same sampling on the same day of collection differed significantly in their study. For PARENTE et al. (2020), although diverse, the milk microbiota shows some similarity in some studies, especially at the phylum and genus level.

The total amount of microorganisms in raw, pasteurized and sterilized milk shows that refrigerated raw milk has the highest number of microorganisms (29,334), followed by pasteurized milk (14,674) and sterilized milk (7,393). This represents a reduction of 49.97% of the total microorganisms from refrigerated raw milk to pasteurized milk and 74.79% from refrigerated raw milk to sterilized milk. This decrease indicates that milk processing steps are being effective in the general decrease of the present microorganisms. The decrease is necessary for milk to have microbiological levels safe to human health (ROSENBERG, 2020). However, the beneficial food microbiota also ends up being eliminated or decreased considerably, such as important probiotic microorganisms.

Regarding the frequency of microorganisms in the samples, it can be observed that a single microorganism, *Sphingomonas echinoides*, was present in all six samples. Despite being the most frequent microorganism, *S. echinoides* is not a microorganism abundant in the samples, and the sum of all sequences obtained is nine, that is, four of the six samples analyzed presented only one sequence of this microorganism. VRIES et al. (2018), this microorganism is a biofilm-former and can metabolize a wide range of substrates. These characteristics may be related to the fact that this microorganism appears in all samples, even with negligible abundance, because the temperature variations of the samples at the time of collection were from 1.2 to 30 °C. Nine other microorganisms were present in five of the six samples analyzed: *Bacillus cereus* group, *Enterobacter bacterium, Enterobacter hormaechei, Kluyvera intermedia, Streptococcus macedonicus, Acinetobacter johnsonii, Kurthia gibsonii, Staphylococcus epidermidis, Acinetobacter baumannii*.

The most abundant microorganisms found in the three types of milk analyzed in this study are shown in Fig. 1a. The microorganism with the highest number of sequences found was *K. gibsonii* (9,569), followed by *Priestia megaterium*, (8,696) and *S. macedonicus* (8,459). Additionally, *K. gibsonii, A. baumannii, B. cereus* group and *E. bacterium* were present in five of the six samples analyzed—they are therefore frequent microorganisms. *Kurthia gibsonii* is in five of the six samples analyzed, besides being abundant in the three types of milk, raw refrigerated, pasteurized and sterilized (Fig. 1).

Table 2. Total of each species sequences found in the samples of refrigerated, pasteurized and sterilized raw milk from the industries of Vale do Taquari (RS).

Species	Total	Species	Total	Species	Total
Kurthia gibsonii	9569	*Comamonas aquatica*	110	*Pseudomonas fragi*	24
Priestia megaterium	8696	*Streptococcus oralis*	102	*Streptococcus agalactiae*	21
Streptococcus macedonicus	8459	*Morganella morganii*	88	*Prevotella melaninogenica*	19
Enterobacter cloacae	5510	*Pantoea agglomerans*	84	*Providencia alcalifaciens*	19
Bacillus cereus group	5450	*Pseudomonas aeruginosa*	83	*Serratia marcescens*	19
Lysinibacillus sphaericus	3475	*Pseudomonas putida group*	81	*Ralstonia insidiosa*	18
Enterobacter bacterium	2201	*Proteus vulgaris*	79	*Acinetobacter johnsonii*	17
Lactococcus garvieae	1325	*Acinetobacter bereziniae*	66	*Citrobacter braakii*	17
Acinetobacter baumannii	1097	*Enterobacter hormaechei*	66	*Comamonas kerstersii*	17
Aeromonas hydrophila	858	*Lysinibacillus fusiformis*	55	*Streptococcus porcorum*	17
Aeromonas dhakensis	427	*Acinetobacter junii*	52	*Staphylococcus epidermidis*	16
Aeromonas caviae	418	*Proteus mirabilis*	45	*Lactococcus lactis*	15
Acinetobacter haemolyticus	354	*Raoultella ornithinolytica*	42	*Enterobacter ludwigii*	14
Streptococcus equinus	262	*Aeromonas sanarellii*	38	*Shewanella xiamenensis*	13
Enterococcus sp.	238	*Vagococcus fluvialis*	34	*Acinetobacter sp.*	12
Viridibacillus arenosi	206	*Klebsiella oxytoca*	33	*Chryseobacterium oncorhynchi*	12
Streptococcus uberis	173	*Aeromonas veronii*	30	*Granulicatella adiacens*	12
Streptococcus thermophilus	163	*Klebsiella aerogenes*	30	*Kluyvera intermedia*	12
Providencia stuartii	157	*Macrococcus caseolyticus*	28	*Pseudomonas azotoformans*	12
Hafnia alvei	147	*Acetobacter pasteurianus*	27	*Empedobacter brevis*	11
Acinetobacter calcoaceticus	137	*Enterococcus italicus*	25	*Lactococcus piscium*	11
Escherichia hermannii	131	*Acinetobacter nosocomialis*	24	*Streptococcus cristatus*	11
Enterobacter mori	127	*Kluyvera cryocrescens*	24	*Kluyvera ascorbata*	10
Citrobacter freundii	122				

Species found in the samples of industries from Vale do Taquari - RS that obtained a total number of sequences greater than 0.2% (10 sequences) of the total microorganisms and the total sequences found for each species.

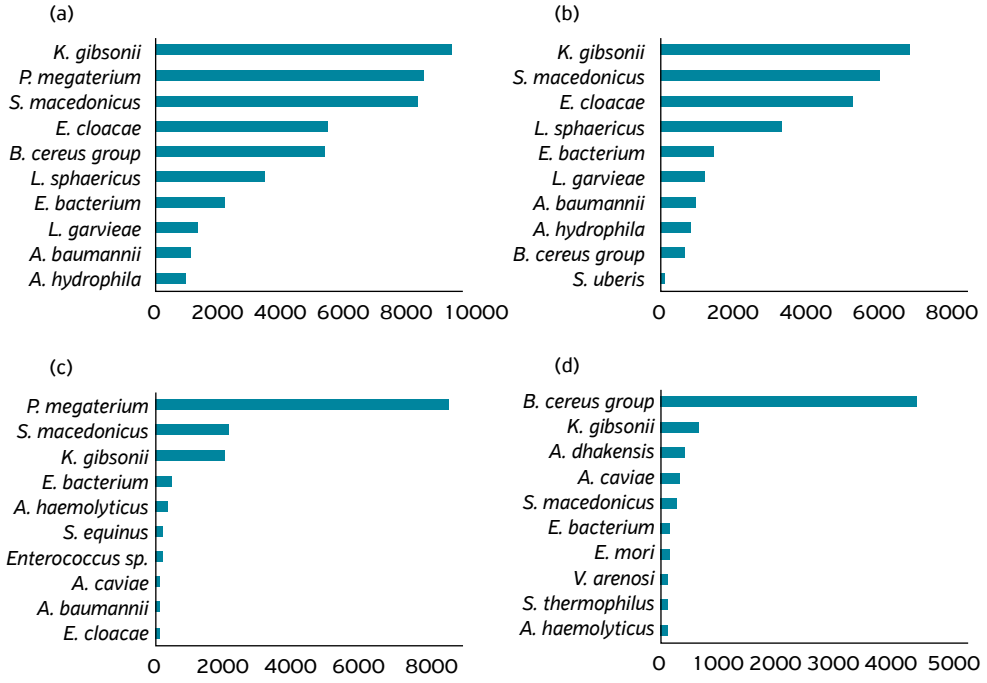

Figure 1. Species and quantities of microorganisms more abundant in (a) the three types of milk; (b) ten most abundant microorganisms in raw refrigerated milk; (c) ten most abundant microorganisms in pasteurized milk; and (d) ten most abundant microorganisms in sterilized milk (d) in the two industries from Vale do Taquari, Rio Grande do Sul, Brazil.

According to SEKOAI et al. (2022), *K. gibsonii* is a gram-positive bacterium of the phylum Firmicutes found in decomposing organic matter, meat products and milk. In their study on the microbial population of wood cutting board used for meat, they found prevalence of *Lactococcus garvieae*, *Weissella hellenica* and *K. gibsonii*. Because this is a microorganism commonly found in foods, such as meat products and milk, its presence in the analyzed samples is within the expected.

The second most abundant microorganism, *P. megaterium*, formerly known as *Bacillus megaterium*, is a Gram-positive endospore-forming microorganism found in seawater, soil and dry foods. It is an industrially relevant species to produce enzymes and vitamins and can also be used for decontamination of toxic waste. *Priestia megaterium* represents a great tool for cell biology studies, mainly as a bacterium promoting growth in plants (BIEDENDIECK et al., 2021).

The third most abundant microorganism in the three types of milk analyzed is *S. macedonicus*, an acid-lactic bacterium commonly used as an initial culture in industrial dairy fermentations due to its ability to rapidly acidify milk and prevent deterioration (EL HATMI et al., 2018).

Acinetobacter baumannii, *B. cereus* group and *E. bacterium*, as mentioned, are frequent, besides being abundant in the samples. To PAKHARUKOVA et al. (2018), *A. baumannii* is one of the main causes of nosocomial infections, having the ability to persist in the environment, as it easily forms biofilms, in addition to possessing resistance to antibiotics. This microorganism has been isolated from various animal, human and environmental sources, where it plays a role in the decomposition of organic matter. The existence of *A. baumannii* in milk samples can be explained by this being a microorganism present in several sources such as animals, besides being a psychrotrophic species, of easily proliferation in refrigerated milk, such as raw and pasteurized milk. The presence of this microorganism, especially in processed milk, deserves attention.

The *B. cereus* group consists of at least 12 Gram-positive, optionally mobile, saprophyte and facultative anaerobic bacteria. This group is common in nature in both endospore forms as in vegetative cells. Endospores are resistant to extreme environmental conditions and are commonly associated with food poisoning. Some species of the *B. cereus* group are psychrotrophic and can grow at temperatures lower than 7 °C, causing concerns for the food industry due to its capacity to cause a deterioration in refrigerated foods, such as raw milk and pasteurized milk (TAKAHASHI et al., 2021). The fact that this microorganism strains are resistant to extreme environmental conditions may explain their abundance and frequency in milk samples from industries in Vale do Taquari, whether in milk refrigerated or exposed to the thermal process, as is the case of pasteurized and UHT.

According to MUENSRITHARAM et al. (2016), *Enterobacter* species are associated with a variety of environmental habitats, usually found in soil and water, in addition to containing the main antibiotic-resistant bacterial pathogens. These microorganisms are not recognized as important food-borne pathogens, but may be found in a wide variety of foods. The species found in milk are usually eliminated by pasteurization, but members of this genus were found in pasteurized milk and milk cream. In the present study, the pasteurized milk showed the presence of *E. bacterium*. The fact that this microorganism is found in several environments, including water, may explain its presence in the milk samples from this study, as many animals use surface water for their drinking or may still ingest this microorganism in their diet.

For being microorganisms capable of causing damage to human health, the presence and abundance of *K. gibsonii*, *A. baumannii, B. cereus* group and *E. bacterium* in samples deserve attention; moreover, *E. bacterium* and *A. baumannii* show resistance to antibiotics and biofilm formation capacities, making it more difficult for the treatment and disposal on the environment. According to MORADI; TAJIK (2017), biofilm formation promotes the growth and survival of pathogenic microorganisms within food processing units and are major risks to public health.

In addition to the microorganisms mentioned, *Lysinibacillus sphaericus*, *L. garvieae* and *A. hydrophila* were also abundant in the total number of analyzed samples, with respectively 3,475, 1,325 and 858 sequences. *Lysinibacillus sphaericus* is a spore-forming bacterium, considered plant growth promoter (RODRÍGUEZ et al., 2019). According to ERACLIO et al. (2017), *L. garvieae* is one of the most important pathogens in the aquaculture sector, for causing infections in fish. This species is found in different types of food, besides colonizing different types of environments. *Aeromonas hydrophila* is a Gram-negative bacterium also found in various aquatic environments, which can cause septicemia in humans. The contamination of milk samples by *L. garvieae* and *A. hydrophila*, microorganisms causing diseases in fish, may be tied to the environment where the animals are raised, as often there are other activities in milk-producing properties, such as fish ponds, and animals can roam near the site or use the water for their drinking (LI et al., 2021).

When analyzing the ten most abundant microorganisms in each type of milk (raw refrigerated milk up to milk sterilized by the UHT process), it can be seen that *K. gibsonii, E. bacterium* and *S. macedonicus* appear as abundant in the three types. By adding the number of sequences of the ten most abundant in each type of milk, it is possible to observe a gradual decrease from the raw refrigerated milk to sterilized milk, as observed in the sum of the total amount

of sequences. The ten most abundant total were 27,294 sequences in raw refrigerated milk, 13,941 in pasteurized milk, and 6,939 in sterilized milk. As already observed in studies such as that of TAPONEN et al. (2019), there is a great diversity in microorganisms observed in the samples of the same type of milk in each of the collected companies. The microorganisms described in this study as the most abundant for each type of milk were almost totally restricted to only one of the industries, except for *S. macedonicus*, in raw refrigerated milk. Therefore, there was no sum of sequences of the most abundant species for milk types.

The most abundant microorganism in raw refrigerated milk was *K. gibsonii* with 6,907 sequences, followed by *S. macedonicus* (6,107, being 1,067 at C1 and 5,040 at C2), *Enterobacter cloacae* (5,339) and *L. sphaericus* (3,402) (Fig. 1b). Seven (70%) of the ten most abundant microorganisms were in the sample from Company 1. *Enterobacter cloacae* is included as a common nosocomial pathogen capable of producing a variety of infections and having resistance to broad-spectrum antibiotics, but its presence in milk is not a concern as the heat treatment of milk by UHT safely inactive members of the Enterobacteriaceae family (ANNAVAJHALA et al., 2019).

When analyzing the ten most abundant microorganisms in pasteurized milk (Fig. 1c), the microorganism with the highest number of sequences was *P. megaterium*, with 8,696, followed by *S. macedonicus* (2,091) and *K. gibsonii* (1,992), being the other seven less expressive sequence numbers, lower than 400. Seven of the ten most abundant microorganisms belonged to C2, but the most abundant microorganism (*P. megaterium*) was present only in the pasteurized milk sample of C1-P. When comparing the abundance in the analyzed samples, it can be seen that *P. megaterium* (with 8,696 sequences) is restricted to pasteurized milk.

In this study, *E. cloacae* and *A. baumannii* appear to be abundant in raw and pasteurized milk. These microorganisms, being found in pasteurized milk cause concern, because although little consumed, this milk is used to produce yogurts and other products in the dairy industries. Additionally, pasteurized milk also presented *Streptococcus equinus*, which is a microorganism belonging to the *Streptococcus bovis/S. equinus* complex, a diverse group of bacteria that includes inhabitants of the gastrointestinal tract of humans and animals, being an opportunistic pathogen found in food (KAINDI et al., 2018).

When analyzing the ten most abundant microorganisms in sterilized milk (Fig. 1d) it was found that the B. cereus group was the most abundant, with 4,743 sequences identified. All other microorganisms obtained a much lower number of sequences, being the second *K. gibsonii*, with 661, and the last placed *Acinetobacter haemolyticus*, with only 83 sequences. The most abundant microorganism, as well as 80% of the total abundant from sterilized milk were present in the sample from C1.

Bacillus cereus group and *A. haemolyticus* were abundant in the samples of raw refrigerated milk and in the sterilized milk samples. The latter was defined as a Gram-negative, strictly aerobic and non-fermentative coccobacillus widely distributed in nature and commonly found in soil, water, and hospitals and constitutes a challenge for public health (BAI et al., 2020). Because it is a microorganism found in soil and water it may have been ingested by the animals and therefore found in the milk samples of the present study.

Milk sterilized by the UHT process also presented sequences of microorganisms such as *Aeromonas dhakensis* (400), *Aeromonas caviae* (321), *Enterobacter mori* (120), *Viridibacillus arenosi* (113) and *Streptococcus thermophilus* (105). *Streptococcus thermophilus*, as well as *S. macedonicus* commented earlier, is a widely known microorganism and used in the food industry. It is a probiotic of lactic acid widely used in dairy products as an initial culture for the manufacturing of cheeses and yogurts (PHILIPPE et al., 2020). *Aeromomas dhakensis* is a pathogen, infection-causing, widely distributed in the environment and causing a variety of infections in humans (CHEN et al., 2017). *Aeromomas caviae* is also a microorganism found in the environment, usually in places with high salinity (CARDOZO et al., 2019). *Viridibacillus arenosi* is an aerobic bacterium that forms spores, psychrotolerant and deteriorating. This microorganism is predominant in foods such as milk (THAKUR et al., 2017). *Enterobacter mori* is a plant pathogenic microorganism, being described as the causing agent by bacterial wilt in *Morus alba* (white mulberry), a serious disease in orchards. This microorganism is not commonly associated with human diseases, but in its study is reported a case of acute extreme otitis, in Austria, whose isolate demonstrated *in vitro* resistance to carbapenems (HARTL et al., 2019).

Heat treatment in milk has the function of eliminating all pathogenic bacteria that can cause infections to consumers, in addition to inactivating enzymes and reducing the total amount of microorganisms, so that it is possible to extend the shelf-life of the product. The UHT processing allows to achieve commercial sterility, with minimal impact on the milk nutritional value (ROSENBERG, 2020). The Ordinance No. 370 (BRAZIL, 1997) brings as microbiological criteria that UHT milk must present up to 100 CFU/mL of mesophilic aerobics. The levels observed in this study are above the maximum limit allowed by the ordinance, but metagenomics is a more sensitive method, which allows the identification of a more accurate number of microorganisms in the samples than the methods used for conventional microbiological analysis.

The presence of *B. cereus* group, *A. haemolyticus*, *A. dhakensis* and *A. caviae* deserves attention because they are microorganisms causing infections in humans and their presence in the samples indicates that the thermal process was not completely efficient. STRÖHER et al. (2021) concluded in their study that UHT milk with lower quality came from raw material with low quality, i.e., raw refrigerated milk with high microorganism count, or high total bacterial count. According to ROSENBERG (2020), inappropriate milk storage has a significant impact on the composition of the microbial community. This impact is greater for species of the *Streptococcus*, *Staphylococcus*, *Macrococcus*, and *Corynebacterium* genera and deteriorating bacteria, such as *Acinetobacter* and *Pseudomonas*, psychrotrophic bacteria that form spores (PARENTE et al., 2020). In this study, several species of the *Streptococcus* and *Acinetobacter* genera were observed in the samples collected in the dairy industries.

Among the ten most abundant in the three types of milk and raw refrigerated milk there are nine common microorganisms. *Priestia megaterium* appears only in pasteurized milk, as already mentioned and *S. uberis* in raw refrigerated milk; the latter being one of the main causes of clinical mastitis worldwide, being considered a barrier in its control due to its epidemiology not being fully understood (TOMAZI et al., 2019). Because it is a mastitogenic microorganism, its presence is common in raw refrigerated milk.

By observing the different types of microorganisms present between raw refrigerated milk and processed milk (pasteurized and sterilized), it can be noticed that the processing is efficient in the elimination of microorganisms such as *L. sphaericus*, *L. garvieae* and *A. hydrophilic* and, in addition to these, sterilization (UHT) is still efficient for *E. cloacae*, *A. baumannii*. Spore-forming microorganisms such as *B. cereus* group, *V. arenosi* and *P. megaterium* may be more difficult to eliminate by the thermal process.

The samples Shannon Diversity Index ranged from 0.2112 in the pasteurized milk sample from company 1 (C1-P) to 2.18 in the raw refrigerated milk sample also from company 1 (C1-R). The indexes of each sample were: raw refrigerated milk: 2.18 (C1-R) and 1.153 (C2 -R); pasteurized milk: 0.2112 (C1-P), 1.919 (C2-P) and sterilized milk: 1,399 (C1-S) and 1,817 (C2-S). The multivariate principal component analysis (Fig. 2) shows that the component 1 (x-axis) explains 33.29% of the data variability and component 2 (y-axis) explains 17.67% of the variability. Together these two components explain 50.96% of the results. Through the analysis of the perceptual map, it can be verified that the samples from company of raw refrigerated milk 1 (C1 – R), sterile milk (C1 – S) and the pasteurized milk sample of company 2 (C2 – P) are correlated. The company 2 sample of raw refrigerated milk (C2 – R) and sterilized milk (C2 – S) also have correlation, indicating similarity in the microorganisms found in both samples and their quantity.

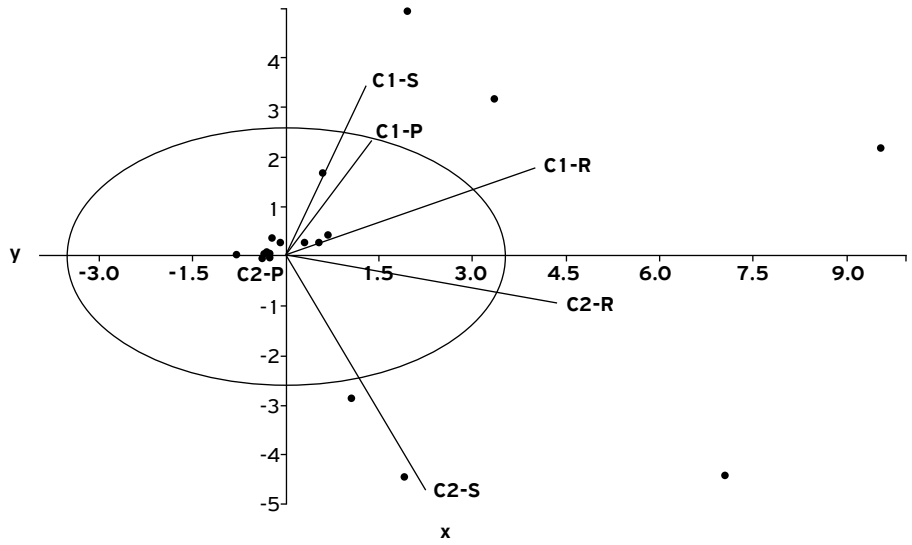

Figure 2. Perceptual map of the multivariate analysis of milk samples collected in industries from Vale do Taquari, Rio Grande do Sul, Brazil. Sort chart using multivariate analysis in Past. C1-R: Raw milk from Company 1; C2-R: Raw milk from Company 2; C1-P: Pasteurized milk from Company 1; C2-P: Pasteurized milk from Company 2; C1-S: Sterilized milk from Company 1; C2-S: Sterilized milk from Company 2.

When analyzing the samples for species diversity, it can be observed that the sample of raw refrigerated milk from company 1 (C1-R) has the largest number of different species with 62 species of microorganisms, followed by the pasteurized milk sample from company 2 (C2-P). The sample with the smallest number of different species is raw refrigerated milk from company 2 (C2-R), with only 17 different species (Fig. 3a).

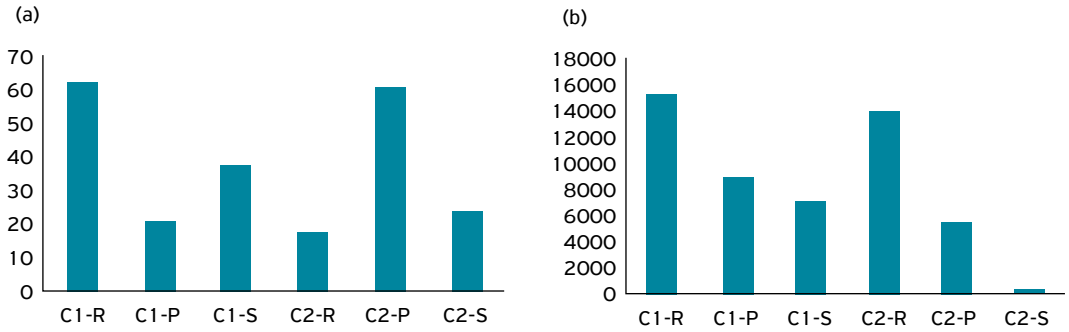

Figure 3. Abundance and diversity of different species found in each milk sample collected in the industries of Vale do Taquari, Rio Grande do Sul, Brazil. (a) Species diversity found in refrigerated, pasteurized and sterilized raw milk samples; and (b) Abundance of species found in refrigerated, pasteurized and sterilized raw milk samples. C1-R: Raw milk from Company 1; C2-R: Raw milk from Company 2; C1-P: Pasteurized milk from Company 1; C2-P: Pasteurized milk from Company 2; C1-S: Sterilized milk from Company 1; C2-S: Sterilized milk from Company 2.

When analyzing the samples separately regarding microbiological abundance, it is possible to notice that the two samples with the highest abundance are of raw refrigerated milk from company 1 and company 2, with a total of 15,384 (C1-R) and 13,950 (C2-R) sequences, respectively. The sterile milk sample from company 1 (C1-S) has greater abundance than the pasteurized milk sample from company 2 (C2-P). However, when the number of sequences in the samples from the same company was analyzed, there was a gradual decrease in the two samples of raw refrigerated milk for the two types of processed milk, pasteurized and sterilized, respectively (Fig. 3b).

CONCLUSION

The analysis of milk collected from the industries in Vale do Taquari showed a significant decrease in the number of microorganisms from raw refrigerated milk to milk processed by pasteurization and sterilization processes (UHT). This indicates that the processes are effective in reducing the total amount of microorganisms, in addition to eliminating microorganisms that cause milk deterioration or problems to the consumer health, such as *L. sphaericus, L garvieae, A. hydrophilic, E. cloacae* and *A. baumannii*. The processed milk demonstrated the presence of microorganisms beneficial to human health, such as *S. thermophilus* and *S. macedonicus*, but also microorganisms considered harmful as the *B. cereus* group, *A. dhakensis, E. bacterium* and *A. haemolyticus*, showing that thermal processes have not been completely efficient. Metagenomics allows the identification of the microbiota present in milk and, consequently, a more accurate evaluation of its quality. These studies can be used to improve the processing steps used by industries and to trace possible contaminant sources. Improving the quality of the milk produced is essential to ensure the health of the consumer.

AUTHORS' CONTRIBUTIONS

Conceptualization: Müller, T.; Rempel, C.; Maciel, M.J.; Lunardi, L. **Data curation:** Müller, T.; Rempel, C. **Formal analysis:** Müller, T.; Rempel, C. **Funding acquisition:** Rempel, C.; **Investigation:** Müller, T. **Methodology:** Müller, T.; Maciel, M.J. **Project administration:** Rempel, C. **Resources, Software:** Müller, T.; Rempel, C.; Maciel, M.J. **Supervision:** Rempel, C.; Maciel, M.J. **Validation:** Rempel, C.; Maciel, M.J. **Visualization:** Rempel, C.; Maciel, M.J. **Writing – original draft:** Müller, T.; Rempel, C.; Maciel, M.J.; Lunardi, L. **Writing – review & editing:** Rempel, C.; Maciel, M.J.

AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article.

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CONFLICTS OF INTEREST

The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

Not applicable.

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Artigo 5 – "Milk microbiota from dairy factories in the central region of Rio Grande do Sul, Brazil"[1]

Microbiota do leite de laticínios na região central do Rio Grande do Sul, Brasil

ABSTRACT

Milk is a food with considerable nutritional value. Brazil is the world's fifth largest producer of this food. Its quality and microbiota are influenced by several factors. The goal of the present study was to determine the microbiota of refrigerated raw milk and processed milks in dairy factories in Taquari Valley - RS, through genetic sequencing. Three types of milk were analyzed in two dairy factories of the region: refrigerated raw milk – which arrives at the dairy factories by tank trucks, pasteurized milk, and Ultra High Temperature (UHT) sterilized milk. The determination of the microbiota of milk was performed by partial sequencing of the 16S rRNA gene. The results showed that refrigerated raw milk has the highest number of microorganisms in the two dairy factories, followed by pasteurized milk and milk sterilized by the UHT process, successively. The processing of dairy factory 2 proved to be more efficient, especially for UHT milk, considerably reducing the microbiota. Eighty-seven species of the Kingdom Bacteria were identified, and the samples showed considerable microbiological diversity, even within the same type of milk. Lactic acid bacteria such as *Streptococcus macedonicus* were found in refrigerated raw milk and pasteurized milk and *Streptococcus thermophilus* in sterilized milk. Harmful species such as *Bacillus cereus group*, *Aeromonas dhakensis* and *Acinetobacter haemolyticus* were found in the UHT milk of both dairy factories.

Index terms:

Microorganisms; microbiota; pasteurized milk; sterilized milk; raw milk.

RESUMO

O leite é um alimento com considerável valor nutricional. O Brasil é o quinto maior produtor mundial desse alimento. A sua qualidade e a microbiota é influenciada por diversos fatores. O objetivo do presente estudo foi determinar a microbiota do leite cru refrigerado e de leites processados em laticínios do Vale do Taquari – RS, por meio do sequenciamento genético. Foram analisados três tipos de leite em dois laticínios da região: leite cru refrigerado, que chega aos laticínios por meio dos caminhões-tanques, leite pasteurizado e leite esterilizado por *Ultra High Temperature* (UHT). A determinação da microbiota do leite foi realizada por meio do sequenciamento parcial do gene 16S rRNA. Os resultados mostraram que o leite cru refrigerado possui a maior quantidade de microrganismos nos dois laticínios, seguido do leite pasteurizado e pelo leite esterilizado pelo processo UHT, sucessivamente. O processamento do laticínio 2 mostrou-se mais eficiente, principalmente para o leite UHT, reduzindo consideravelmente a microbiota. Foram identificadas 87 espécies do

[1] Artigo em análise na Revista Ciência e Agrotecnologia.

Reino *Bacteria* e as amostras mostraram considerável diversidade microbiológica, mesmo dentre o mesmo tipo de leite. Bactérias ácido-láticas como *Streptococcus macedonicus* foram encontradas no leite cru refrigerado e no leite pasteurizado e *Streptococcus termophilus*, no leite esterilizado. Espécies nocivas como *Bacillus cereus group*, *Aeromonas dhakensis* e *Acinetobacter haemolyticus* foram encontrados no leite UHT de ambos os laticínios.

Termos para indexação:

Microrganismos; microbiota; leite pasteurizado; leite esterilizado; leite cru refrigerado.

INTRODUCTION

Milk is an essential food, with nutritional relevance, which makes it one of the main agricultural products (Carvalho et al., 2021). Milk production makes a relevant contribution to the economy and social development, with around 150 million families working in milk production worldwide. Most producers are smallholder farmers in developing countries, and this is the main activity for their livelihood (FAO, 2021).

Brazil is considered the fifth world's largest milk producer and produced 5,815,050 thousand liters in the second quarter of 2021, being Rio Grande do Sul responsible for 885,000 liters (IBGE, 2022). The Taquari Valley region, located in the central region of the state, is responsible for a large part of the state production, with an average of 4,406,428 thousand liters per year and more than one million liters of milk per day, being the third largest milk-producing region (SPGG, 2020). Milk production is one of the core businesses of the economy of the small towns that make up Taquari Valley - RS.

The quality of milk can be influenced by a number of factors, from processing, on milk-producing properties, to beneficiation carried out by dairy factories. In order to avoid contamination, Good Agricultural Practices (GAPs) must be adopted, which consist of a set of activities developed in the rural properties with the goal of ensuring health, well-being and safety of animals, human beings and environment (Ströher et al., 2021). Normative Instruction Number 77, from Ministry of Agriculture, Livestock and Supply (MAPA), regulates the implementation of GAPs in the stages of bovine milk production (Brazil, 2018b).

MAPA's Normative Instruction Number 76 provides information on the identity and quality characteristics that refrigerated raw milk, pasteurized milk and type A

pasteurized milk must present. Refrigerated raw milk is the one produced on rural properties, refrigerated and destined to milk and dairy establishments. Pasteurized milk is the fluid milk subjected to one of the pasteurization processes stated in current legislation, automatically packaged in a closed circuit and intended for direct human consumption (Brazil, 2018a). UHT milk, on the other hand, is understood as homogenized milk, which has been subjected, for two to four seconds, to a temperature of 130º C (Brazil, 1997).

In order to be consumed, the refrigerated raw milk needs to go through the beneficiation processes carried out in the dairy factories. The dairy industry is responsible for ensuring the safety and quality of milk for consumers. The core processes for obtaining a quality product are the cooling and the beneficiation of milk (Machado et al., 2017). The cooling of raw milk must occur at temperatures up to 5° C and remain below that until it arrives at the dairy factory, where it will be used in the production of different types of milk or dairy products (Brazil, 2018a). The type of milk produced depends on the heating process that was applied: pasteurization or UHT. These heating processes eliminate pathogens and increase shelf life in closed packages. Pasteurized milk must be stored at refrigeration temperature (4° C to 7° C) and has a shelf life of about two weeks, whereas UHT milk can be stored at room temperature and lasts up to twelve months in a closed bottle (Machado et al., 2017).

Milk microbiota can be influenced by several factors, whether endogenous or environmental, and may be composed of microorganisms that are beneficial to human health, or yet deteriorating or pathogenic. The typical composition of bovine milk microbiota was shown to be heterogeneous and characterized by an abundance of lactic acid-producing bacteria (LAB), of the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Leuconostoc* and *Enterococcus*, besides psychotrophic bacteria such as *Pseudomonas*, *Acinetobacter* and *Aeromonas*. More recent and more sensitive methods than traditional microbiological methods have revealed the presence of anaerobic bacteria, such as *Bacteroides*, *Faecalibacterium*, *Prevotella* and *Catenibacterium*, whose origin may be related to fecal contamination events (Tilocca et al., 2020).

Milk microbiota studies have been used to assess and improve animal health and ensure product quality, as well as safety in consumption (Yap et al., 2020).

Metatranscriptome and metagenome sequencing are becoming the most used procedures to decipher the genomic potential of the entire microbiome in foods (Ferrocino; Ranstsiou; Cocolin, 2021). This field of research comprehend molecular genetics and microbial ecology, with the goal of verifying the total content of microorganisms present in the analyzed samples. By detecting the total amount of the microbiota, metagenomics provides the opportunity to reveal microbiological richness that could not be previously observed (Sudarikov; Tyakht; Alexeev, 2017). Metagenomics is a useful tool for the dairy factories as it promotes a thorough diagnosis of the quality of the milk produced, and can be used to improve milk beneficiation processes.

The goal of the present study was to determine the microbiota of refrigerated raw milk and processed milks in dairy products in the central region of Rio Grande do Sul, in municipalities belonging to Taquari Valley. Analyzes were performed by partial sequencing of the 16S rRNA gene.

MATERIAL AND METHODS

The study was carried out in the Taquari Valley region, Rio Grande do Sul, Brazil. A total of six samples were collected in two dairy factories, in two tows, being one sample of refrigerated raw milk from the tank truck, one of pasteurized milk and one of UTH sterilized milk in each of the industries.

The dairy factories received the denomination D1 (Dairy 1) and D2 (Dairy 2) and the types of milk received their initial, being “R” for raw milk, “P” for pasteurized milk and “S” for sterilized milk. At the time of collection, the sample temperature was measured using an Incoterm thermometer (model 5135). The collections were carried out with 100 mL sterilized plastic bottles, all hygiene precautions were followed, and the samples were placed in a styrofoam box with ice, which kept the temperature of the samples below 12° C.

The high-performance sequencing of the V3/V4 regions of the 16S ribosomal gene was used to identify the bacteria present in the samples. Amplification was performed with primers for region V3-V4 of the rRNA gene 16S, 341F (CCTACGGGRSGCAGCAG), and 806R (GGACTACHVGGGTWTCTAAT). PCR reactions were performed in triplicates, with the conditions: 95° C for 5 min, 25 cycles

of 95° C for 45s, 55° C for 30s and 72° C for 45s and a final extension of 72° C for 2 minutes.

The MiSeq Sequencing System equipment was used to sequence the genomic libraries. For single-end sequencing, the V2 kit with 300 cycles was used. The sequences were analyzed using the Sentinel pipeline. In the Sentinel pipeline, fastq files are evaluated for Phred quality (PQ) using the FastQC v.0.11.8 program. Therefore, fastq files are subjected to low quality primers and sequence trimming (Phred < 20). The software used for this purpose was built in Python v.3.6, which is inspired by the features of the BioPython project. For paired-end data, before the trimming step, two pairs of files (R1 and R2) are merged into a single file using pandaseq v.2.11. Clusters with abundance less than two were removed from the analysis, as such structures are usually related to chimera sequences. Taxonomic identifications are performed with BLASTn v.2.6.0 (Altschul et al., 1990), using a proprietary or public database as reference. Regarding the definition of a species, among the 20 hits returned for each cluster, a Python instruction evaluated whether one of the three requirements would be met by the hits: 1) higher bit-score; 2) lower evalue; and 3) taxonomies with greater representation.

Data were tabulated using Excel. The average and standard error of the number of species were calculated using Excel. The Q-square test was performed using Bioestat, in order to verify statistical differences in the species found in the six analyzed samples.

RESULTS AND DISCUSSION

The analysis of samples of refrigerated raw milk, pasteurized milk and sterilized milk from the dairy factories in Taquari Valley (RS) showed the presence of 51,401 sequences of Kingdom Bacteria, divided into three phyla: *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, nine classes, 15 orders, 21 families, 41 genera and 87 species.

In relation to the total number of microorganisms, it is possible to observe that the samples with the highest number of microorganisms were those of refrigerated raw milk, followed by samples of pasteurized milk and sterilized milk. The sample with the highest number of microorganisms was the refrigerated raw milk sample from Dairy 1, with 15,384 sequences, followed by the refrigerated raw milk sample from Dairy 2, with

13,950 sequences. Dairy 1 also showed the highest number of microorganisms compared to Dairy 2 for processed milks (pasteurized and sterilized). Pasteurized milk from Dairy 1 had 9,052 sequences and pasteurized milk from Dairy 2 had 5,622 sequences. The sterilized milk showed an even greater difference, and in Dairy 1 the total number found in the sample was 7,090 sequences, and in Dairy 2 it was only 303 sequences. In addition, the sterilized milk from Dairy 1 has a higher number of microorganisms than the pasteurized milk from Dairy 2.

The number of microorganisms in the samples showed a reduction from the refrigerated raw milk to the pasteurized milk and to the sterilized milk, successively, in the samples of the two dairy factories. In Dairy 1, there was a reduction of 41.15% in the total amount of microorganisms from refrigerated raw milk to pasteurized milk and 53.91% from refrigerated raw milk to sterilized milk. In Dairy 2 it is possible to observe an even greater reduction, from 59.69% of refrigerated raw milk to pasteurized milk and from 97.83% of refrigerated raw milk to sterilized milk. This shows that the beneficiation processes have been efficient in reducing the microbial load of raw milk in the two dairy factories analyzed, but Dairy 2 shows a much higher efficiency, especially in its UHT processing.

Using the Chi-square test, it is possible to observe that there is a significant variation in the species of microorganisms found in the samples of dairy factories from Taquari Valley. This statistical difference occurs when comparing the three types of milk analyzed: raw refrigerated, pasteurized and sterilized, from the same dairy factory, and also when the two dairy factories are compared in relation to the three types of milk. Moreover, when comparing the same type of milk from the two dairy factories, there is also a statistical difference ($p < 0.001$).

The total average of microorganisms found in the analyzed samples was 590.8 microorganisms and the standard error was 1,836.2, due to the significant differences in the number of each species of microorganism found in the samples (Figure 1). These differences range from 8,696 sequences in one sample (pasteurized milk from Dairy 1) to zero in the other samples. The average number of microorganisms in the samples ranged from 176.8 in the refrigerated raw milk sample from Dairy 1 to 3.5 microorganisms in the sterilized milk sample from Dairy 2, and the standard error ranged from 99.95 in the pasteurized milk sample from Dairy 1 to 1.85 in the sample

of sterilized milk from Dairy 2. The biggest difference between the averages, as well as between the total of microorganisms found, is observed for the sterilized milk, which has 81.5 sequences of microorganisms in Dairy 1 and 3.5 sequences of microorganisms in the Dairy 2.

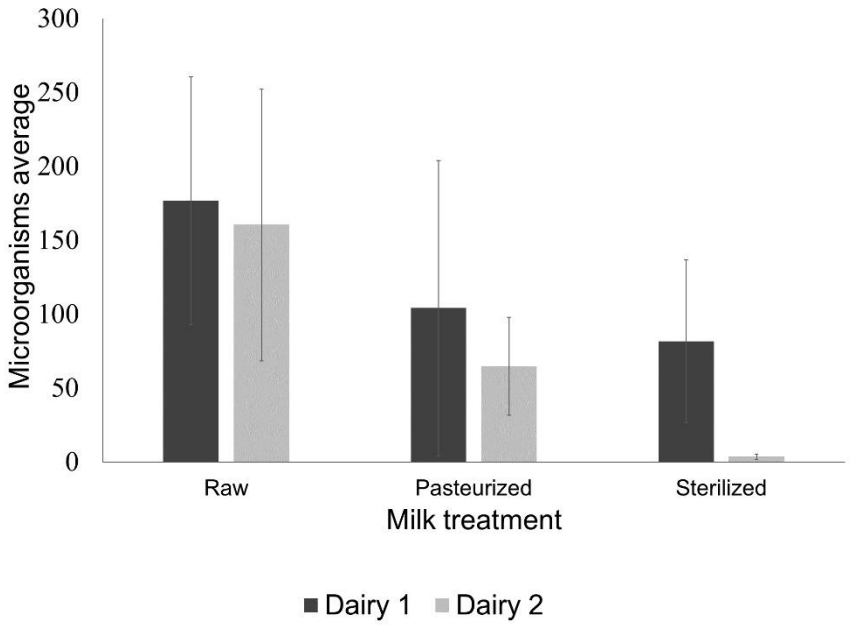

Figure 1. Average number of sequences of microorganisms found in samples of refrigerated raw milk, pasteurized milk and sterilized milk in the dairy factories of Taquari Valley – RS.

The reduction of the microbiota occurs mainly through thermal processes, which have the function of eliminating all pathogenic bacteria present in the food, besides inactivating the enzymes produced (Qianqian et al., 2020). This reduction in the number of microorganisms is necessary so that the milk can be ingested without causing harm to the consumer's health. However, in this reduction process, there is also a decrease or elimination of beneficial microbiota from the food, such as probiotic microorganisms, e.g. LAB. According to Markowiak and Śliżewska (2017), probiotics have numerous advantageous functions for human health. The main advantage is the effect on the development of the organism's own microbiota, in order to ensure the proper balance between pathogens and beneficial bacteria, necessary for its functioning. Human probiotic microorganisms mainly belong to the genera: *Lactobacillus*, *Bifidobacterium*, *Lactococcus*, *Streptococcus* and *Enterococcus*. For Viscard et al. (2020), the ingestion of food with present living microorganisms will

promote a direct gain to health, known as the probiotic effect, or indirect (intake of microbial metabolites), known as the prebiotic effect.

According to current legislation, the total bacterial count (TBC) in refrigerated raw milk must not exceed 900,000 CFU/mL in the tank truck that arrives at the dairy factory (Brazil, 2018a). For UHT milk, the maximum amount allowed is 100 CFU/mL of mesophilic aerobes (Brazil, 1997). Pasteurized milk does not have established TBC or mesophilic levels. The levels observed in this study are above the maximum limit allowed for UHT milk in both dairy factories, and even the sample of sterilized milk from Dairy 2, with 303 sequences, has three times more microorganisms than the maximum allowed. It should be noted that metagenomics is a more efficient method than traditional microbiological methods and that it allows a deeper analysis of milk samples in relation to the existing microbiota, not existing levels established by current legislation for data obtained through these methods.

According to Mariotto et al. (2020), the quality and shelf life of processed milk are directly related to the initial microbial load present in refrigerated raw milk that arrives at the dairy factory. According to Rosenberg (2020), the thermal processing of milk allows reaching commercial sterility. The quality and stability of UHT milk are affected by the storage and cooling conditions of the milk. In order to guarantee a quality product, it is necessary that the milk arrives at the dairy factory with a low somatic cell count (SCC) and a low count of psychrotrophic microorganisms.

The five main genera found in milk samples from the Taquari Valley dairy factories were: *Bacillus* (14,146), *Kurthia* (9,569), *Streptococcus* (9,222), *Enterobacter* (5,747), *Lysinibacillus* (3,530) and *Aeromonas* (1,776). Genres such as *Streptococcus*, *Staphylococcus* and *Aerococcus* may be related to milk from animals with mastitis. The genus *Bacillus* is composed of gram-positive psychrotrophic microorganisms, associated with spoilage, frequently reported as thermoduric and thermophilic sporulated microorganisms, which directly influence the shelf life of pasteurized milk (Ribeiro Junior et al., 2018).

The main genera in the refrigerated raw milk sample from Dairy 1 (D1-R) were: *Kurthia*, *Streptococcus*, *Lactococcus*, *Acinetobacter*, *Aeromonas*, *Bacillus*, *Providencia*, *Enterobacter*, *Escherichia*, *Enterococcus* and *Hafnia*. In pasteurized milk (D1-P) they were: *Bacillus* and *Acinetobacter* and in sterilized milk (D2-S) they were:

Bacillus, *Aeromonas*, *Kurthia*, *Streptococcus*, *Enterobacter* and *Viridibacillus* (Figure 2).

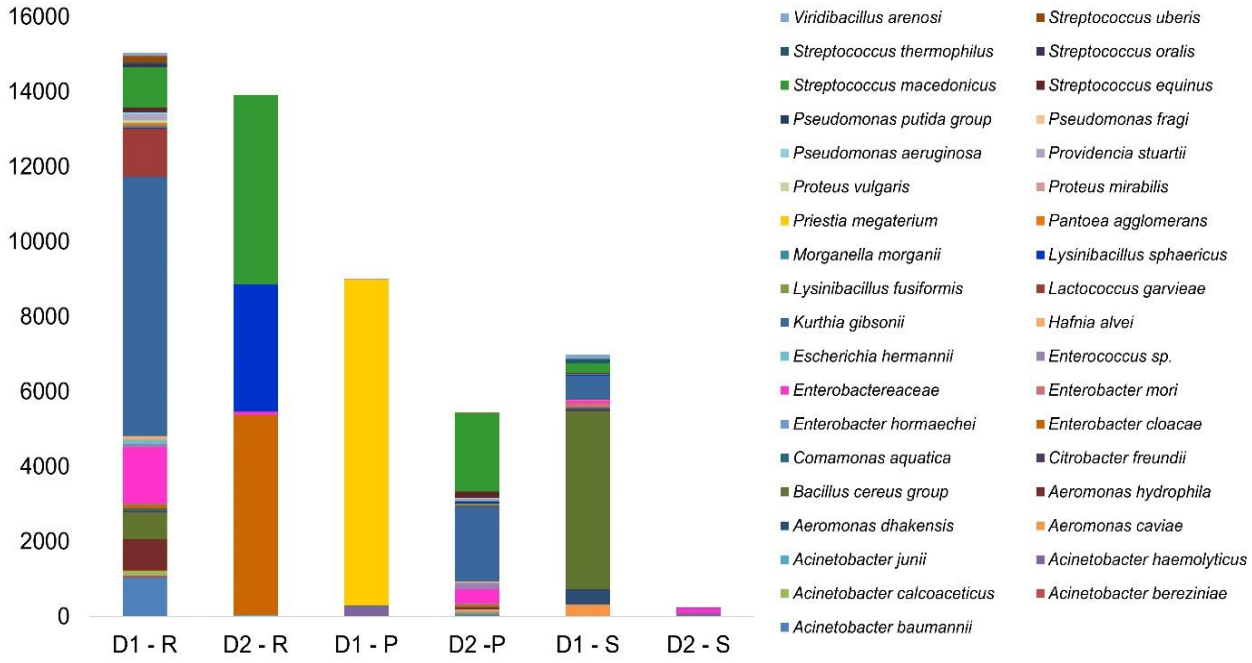

Figure 2. Main species and family *Enterobactereaceae* found in milk samples from dairy products in the Taquari Valley. D1-R: refrigerated raw milk sample from Dairy 1; D2-R: refrigerated raw milk sample from Dairy 2; D1-P: pasteurized milk sample from Dairy 1; D2-P: pasteurized milk sample from Dairy 2; D1-S: sterilized milk sample from Dairy 1; D2-S: sterilized milk sample from the Dairy 2.

The main genera in the refrigerated raw milk sample from Dairy 2 (D2-R) were: *Enterobacter*, *Streptococcus* and *Lysinibacillus*. In pasteurized (D2-P) they were: *Streptococcus*, *Kurthia*, *Enterococcus*, *Aeromonas*, *Acinetobacter*, *Enterobacter* and *Lysinibacillus* and in sterilized milk (D2-S) they were: *Acinetobacter*, *Lactococcus* and *Pseudomonas*.

Taponen et al. (2019) found great diversity in samples analyzed in their study. In addition, studies on the milk microbiome do not provide a minimum limit of sequences that must be observed for each species found in the analyses. Parente, Ricciardi and Zotta (2020), reported 25 genera found in milk samples, which include psychrotrophs such as *Acinetobacter*, *Chryseobacterium*, *Pseudomonas* and *Psychrobacter*, bacteria common to the intestinal microbiota of the genera *Atopostipes*, *Bacteroides*, *Romboutsia*, *Christensenellaceae*, *Clostridium*, *Rikenellaceae* and

Ruminococcaceae, bacteria common to udders of animals such as *Staphylococcus*, *Aerococcus*, *Turicibacter*, *Streptococcus*, *Facklamia*, *Corynebacterium* and *Bacillus*, in addition to beneficial microorganisms of the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Staphylococcus* and *Corynebacterium*. Comparing data from different studies is difficult due to the lack of standard operating procedures, in addition to the absence of a well-structured database.

According to Ferrocino, Ranstsiou and Cocolin (2021), the central microbiota of dairy products is composed of *Lactococcus, Leuconostoc*, *Enterococcus* and *Streptococci*. In smaller numbers, populations of pathogenic microorganisms occur, and there is a relationship with the state of animal health (e.g. mastitis) and the environment where the animals live (season, farm and temperature).

Considering the total number of species found in the samples, 50 species had a total incidence of less than 1% (51 sequences) of the total sequences (51,401), remaining 37 main species, which are represented in Figure 2. The main species of microorganisms found in raw milk from Dairy 1 (D1-R) were: *Kurthia gibsonii* (6,907 sequences), *Lactococcus garvieae* (1,298), *Streptococcus macedonicus* (1,067), *Acinetobacter baumannii* (1,033), *Aeromonas hydrophila* (839), *Bacillus cereus group* (701) and *Streptococcus uberis* (164). These seven microorganisms add up to 12,000 sequences (78.06%), of the 15,384 sequences present in the sample. Another six microorganisms had sequence numbers between 100 and 150.

K. gibsonii represents 44.89% of the total sequences in the D1-R sample. This microorganism belongs to the phylum *Firmicutes*, being commonly found in decomposing organic matter. Ribeiro Junior et al. (2018) found *K. gibsonii* as one of the main spoilage psychrotrophic species in refrigerated raw milk samples. Gram-positive spoilage microorganisms are often related to the initial and desirable microbiota of milk and their presence is influenced by the environment, animal feed and possible infections of the mammary gland.

L. garvieae is a fish pathogen responsible for lactococcosis, a hemorrhagic septicemia with a high mortality rate and economic impact on fish farms around the world. This microorganism is also considered an emerging human pathogen, being found in the oral cavity, tonsils, rumen, intestines and feces of healthy and sick warm-blooded animals (Thiry et al., 2021). *L. garvieae* is a gram-positive, catalase-negative,

facultatively anaerobic cocco that utilizes carbohydrates to produce lactic acid often found in refrigerated raw milk (Tariq et al., 2020).

According to Tarrah et al. (2018), *S. macedonicus*, the third main microorganism found in the D1-R sample, along with *Streptococcus thermophilus*, are two known species of the genus *Streptococcus* widely used as starter cultures to rapidly reduce the pH of foods, thus inhibiting the development of pathogenic microorganisms. In their study, *S. macedonicus* showed better growth rates under all pH conditions, indicating this species as a possible substitute for *S. thermophilus* in the production of certain foods. LABs of the *Streptococcus* genus, such as *S. macedonicus*, have the potential to be used as biofortified crops for human nutrition (Viscard et al., 2020).

A. baumannii is a species of the genus *Acinetobacter*, commonly found in the microbiota of skin and mouth. This bacterium is a gram-negative coccobacillus, being one of the most emerging species in the world. *A. baumanni* can be found in several foods such as fruit, vegetables and raw milk, as well as dairy products (Elbehiry et al., 2021). For Wareth et al. (2020), this microorganism causes a series of severe infections in the skin and soft tissues, in the urinary and respiratory tracts and in the bloodstream. In addition, isolates collected from milk powder samples in Germany showed wide varieties of *β-lactamase* genes (which produce antibiotic resistance). *A. baumannii* was recently detected as one of the pathogenic bacteria of some fish species such as *Ictalurus punctatus* and *Channa striatus* (Malick et al., 2019). Numerous properties have ponds for fish farming, which may explain the presence of this microorganism in refrigerated raw milk.

B. cereus group is a psychrotrophic, gram-positive, facultative anaerobic microorganism, pathogenic in nature and frequently isolated from food. *B. cereus* is efficient in spore formation and these spores may have high heat resistance. This microorganism is commonly isolated in soil and food samples, and thermal processes, such as pasteurization, are not efficient enough to inactivate its spores. *B. cereus* can cause food poisoning that causes vomiting and diarrhea (Kwon et al., 2022). According to Rodrigues et al. (2017), spore-forming microorganisms are a concern. Raw milk is a source of endospores produced by mesophilic and thermophilic microorganisms and spore-forming psychrotrophic bacteria.

A. hydrophila is a gram-negative bacterium, also found in many aquatic environments, that can cause septicemia in humans. This microorganism is one of the main pathogens that can infect most farmed fish (Li et al., 2021). *S. uberis* is a gram-positive, mastitic, environmental pathogen commonly found in manure. This microorganism is cocci-shaped and occurs in pairs or chains. This bacterium has a membrane-bound special protein, which plays a central role in adherence to bovine mammary epithelial cells (Mihklepp et al., 2019).

K. gibsonii and *B. cereus* group, both psychrotrophic species, along with *S. macedonicus*, a probiotic species, represent 56.37% of the total microorganisms in the sample. According to Tilocca et al. (2020), raw milk has a microbiota with a high diversity of LAB and active microorganisms in the prevention of human pathogens. Raw milk also contains bacteriocins and antimicrobial compounds of bacterial origin, besides several antagonistic biomolecules active in preventing the growth of pathogenic microorganisms. However, pasteurization inactivates or reduces the available concentration of these compounds.

The main microorganisms found in pasteurized milk from Dairy 1 (D1-P) were *Priestia megaterium (8,696), Acinetobacter haemolyticus* (271) and *Pseudomonas fragi* (21). Another 14 microorganisms had an incidence lower than ten sequences. *P. megaterium* represents 96.06% of the microorganisms present in the sample (9,052 sequences in total), and this microorganism was restricted to the D1-P sample.

P. megaterium, formerly called *Bacillus megaterium*, is a gram-positive, endospore-forming microorganism found in seawater, soil, and dry foods. *Bacillus* species such as *P. megaterium* are commonly found in soils and are members of the microbiome of many plant hosts around the world. These microorganisms produce a wide range of bioactive compounds that are involved in promoting plant growth and antiphytopathogenic activities. One of the most important characteristics of *Bacillus* strains is the ability to form spores, thus increasing their ability to resist to a wide range of stress conditions and enabling their application as plant growth-promoting bacteria (PGPB) (Nascimento et al., 2020). This microorganism is also used for the production of penicillin G acylases (PGAs), enzymes used in Biotechnology for the production of lactam antibiotics (Kubiak et al., 2021). *P. megaterium* is restricted to the D1-P sample, and this is the second most abundant microorganism when observing the grand total

of the six samples. However, the first microorganism, *K. gibsonii*, with 9,569 sequences in total, was found in five of the six samples analyzed, being abundant in three.

A. *haemolyticus*, present in smaller numbers, belongs to the genus *Acinetobacter*, which are emerging species as clinically relevant pathogens that can cause a wide range of infections. *Acinetobacter spp*. were previously known as environmental species, which have recently proved their potential to cause infection in humans (Malick et al., 2019). Sharma et al. (2017) carried out a study with an extracellular polymer with mucoadhesive properties produced by *A. haemolyticus* and demonstrated antimicrobial potential against both gram-positive and gram-negative pathogens under in vitro conditions.

P. fragi has an insignificant incidence in the sample, representing only 0.23% of the total sequences. This microorganism is a spoilage often found in kinds of meat, including beef, chicken, pork, lamb and fish. *P. fragi* forms biofilms under refrigerated temperature conditions used in food industries. Biofilms protect resident bacteria from aggression and bad environmental conditions, including desiccation, radiation, predation and antimicrobial compounds. Biofilm formation makes *P. fragi* able to survive to stressful environmental conditions (Wickramasinghe et al., 2017). Pinto Junior et al. (2017) reported in their study that thermostable peptidases produced by *P. fragi* resist to the UHT process, accelerating the milk deterioration and affecting the quality of cheese processed in dairy factories.

Pasteurized milk from Dairy 1 (D1-P), despite having a large number of microorganisms, does not represent a potential risk to consumer health, since almost all microbiological samples are composed of *P. megaterium*, a species not considered harmful.

The main microorganism found in the sterilized milk of Dairy 1 (D1-S) was *B. cereus group* (4,743). Another seven microorganisms had sequence numbers above one hundred: *K. gibsonii* (661), *Aeromonas dhakensis* (400), *Aeromonas caviae* (321), *S. macedonicus* (253), *Enterobacter mori* (120) and *Viridibacillus arenosi* (113) and *S. thermophilus* (105). Considering the total number of microorganisms in the D1-S sample (7,090 sequences), *B. cereus group* represents 66.89%.

A. dhakensis and *A. caviae* are *Aeromonas* species frequently associated with fish diseases (Azzam-Sayuti et al., 2021). According to Liang et al. (2021), A.

dhakensis is a gram-negative waterborne pathogen that can cause gastroenteritis, being considered more virulent than other *Aeromonas* species. According to Sekizuka et al. (2019), *A. caviae* is one of the *Aeromonas* species most adapted to saline water environments, predominant in estuaries and which has often been isolated from environmental sources, such as sewage treatment plants.

E. mori is a microorganism of the *Enterobacter* genus, gram-negative, rod-shaped, non-spore-forming bacteria belonging to the *Enterobacteriaceae* family. This genus is widely distributed in nature and some species function as plant growth-promoting bacteria, while others are recognized as opportunistic pathogens causing several types of infections in humans. *E. mori* is a microorganism responsible for causing diseases in mulberry trees (Zhang et al., 2021).

V. arenosi is a spoilage spore-forming bacterium prevalent in milk (Sun; Atkinson; Zhu, 2021). According to Buehler et al. (2018), the genera *Bacillus* and *Paenibacillus* are the most common psychotolerants linked to the deterioration of dairy products, decreasing the shelf life of these foods. Members of the genera *Bacillus*, *Paenibacillus* and *Viridibacillus* have been isolated throughout the dairy production chain, including soil, silage, feed concentrate and milking equipment, in addition to raw milk and pasteurized milk. *V. arenosi* have the potential to produce enzymes that cause off-flavors and coagulate the final dairy product and therefore decrease the quality of the product.

S. thermophilus, as well as *S. macedonicus,* is used as a starter culture for the production of dairy foods. This microorganism is associated with other lactic bacteria of the genera *Streptococcus*, *Lactococcus* and *Lactobacillus* and is used for the production of fermented dairy products around the world. The proteolytic system of *S. thermophilus* is involved in the release peptide sequences of caseins and whey during lactic acid fermentation (Rodríguez-Serrano et al., 2018).

P. megaterium, found in pasteurized milk, and *B. cereus group* and *V. arenosi*, in sterilized milk from Dairy 1, may have their presence explained because they are spore-forming species. According to Ryu et al. (2020), spores are common contaminants of food products, and their presence can cause food spoilage and even consumer illness. In addition, spores survive heat sterilization, used in dairy processing, and later form biofilms on dairy equipment, contaminating the final

products. Microorganisms such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Campylobacter jejuni* and *B. cereus group*, not only cause food poisoning, but also have a major influence on the acidification and spoilage of milk through the production of lipases and proteases. Milk producing properties are complex environments with a great diversity of microbial ecosystems and this has an overall impact on the microbial ecosystem found in milk.

The sterilized milk sample from Dairy 1 (D1-S) showed a high number of microorganisms when compared to the total found in Dairy 2. Species considered beneficial, psychrotolerant or probiotic such as *S. thermophilus*, S*. macedonicus*, K. gibsonii, *E. mori* and *V. arenosi* together represent 17.65% of the total number of microorganisms. However, microorganisms considered harmful, such as *B. cereus group*, *Aeromonas dhakensis* and *Aeromonas caviae*, represent 77.06% of the sample.

The refrigerated raw milk from Dairy 2 (D2-R) basically presents three main microorganisms: *Enterobacter cloacae* (5,339), *S. macedonicus* (5,040), *Lysinibacillus sphaericus* (3,402), in fourth place comes *Acinetobacter junii* with only 29 sequences. The other microorganisms showed a number of sequences close to ten. From the total of sequences of microorganisms found in the sample, which was 13,950, the three main ones, *E. cloacae*, *S. macedonicus* and *L. sphaericus,* represent 38.27%, 36.12% and 24.38%, respectively, and 98.78% in total. There are two microorganisms in common with the raw milk sample from Dairy 1: *S. macedonicus* and *E. cloacae*, with 110 sequences in the D1-R sample.

E. cloacae is a member of the *Enterobacteriaceae* family. *Enterobacter* species are members of the ESKAPE group, initially described in 1960. There are 18 *Enterobacter* species. They are gram-negative, rod-shaped, generally motile, non-spore forming, oxidase-negative and facultative anaerobes. Foodborne Enterobacter contaminations have been found in pasteurized milk and dehydrated dairy products (Skinnader et al., 2022). Ibrahim, Saad and Hafiz (2021) reported finding several species of this family in powdered milk analyzed in their study, including *E. cloacae*. According to Odenthal, Akineden and Usleber (2016), who also found *E. cloacae* in their study of refrigerated raw milk in Germany, this is not necessarily a public health

concern, as UHT heat treatment of milk safely inactivates members of the *Enterobacteriaceae* family.

Milk contamination by gram-negative microorganisms such as *A. baumannii* (D1-R), *A. haemolyticus* (L1-P) and *E. cloacae* (D2-R) is usually associated with a humid environment and with equipment and water used to clean the milk collection and storage systems (Ribeiro Junior et al., 2018).

L. sphaericus is a gram-positive, mesophilic microorganism, generally found in soil. This microorganism has been studied as an alternative for the control of the *Culex quinquefasciatus* mosquito (Guo et al., 2021). A. *junii* along with *Acinetobacter johnsonii*, *Acinetobacter lwoffii* and *Acinetobacter pittii* were recorded as nosocomial pathogens. *A. junii* is a microorganism found in different natural aquatic environments and is responsible for causing septicemia in humans, being also found in studies with sick fish (Malick et al., 2019). However, the incidence of this microorganism in the sample is not relevant, representing only 0.20%.

The refrigerated raw milk sample from Dairy 1 (D1-R) has the highest species diversity, with sixty-two different species, and the refrigerated raw milk sample from Dairy 2 (D2-R) has the lowest species diversity, with only seventeen different species. According to Metzger et al. (2018), the microbiota of healthy milk usually has greater richness and diversity than the microbiota of milk from glands with mastitis, even in mammary glands from the same cow. A study of teat microbiota, in which researchers collected the first milk samples and teat canal swabs, revealed that diversity was greater also in samples from healthy quarters, who had never had clinical experience of mastitis, compared to quarters that had any case of clinical mastitis. These studies cannot yet determine whether changes in the microbiota precede the development of mastitis, or whether the influx of leukocytes into the mammary gland causes changes in the microbiota.

Pasteurized milk from Dairy 2 (D2-P) showed the microorganisms *S. macedonicus* (2091), *K. gibsonii* (1992), members of the *Enterobacteriaceae* family (392), *Streptococcus equinus* (151) and *Enterococcus sp*. (150) in their main microbiota, and another twenty-five microorganisms had sequence numbers lower than 80 and higher than ten. *S. macedonicus* represents 37.19% of the microorganisms present in the sample (5,622) and *K. gibsonni* represents 35.43%.

The microorganisms belonging to the *Enterobacteriaceae* family are gram-negative, non-spore-forming bacilli and are related to aspects such as the deterioration of various foods. They are associated with intestinal changes and can be found in natural environments. Members of this family most commonly found in dairy products include *Escherichia*, *Salmonella*, *Shigella*, *Yersinia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus*, *Edwardsiella*, *Erwinia*, *Morganella*, and *Providencia* (Singh; Anand, 2020).

S. equinus, synonymous of *Streptococcus bovis* and currently recognized as *S. bovis/S.equine* complex (SBSEC), is commonly found in the digestive tract of humans and ruminants. SBSEC produce lactic acid when growing rapidly with amounts of non-fibrous carbohydrates and are considered important agents of rumen acidosis. The bacterial community in the rumen is highly dependent on the type of feed ingested and diets with a high concentrate content increase the level of carbohydrates, promoting the proliferation of lactic acid-producing amylolytic bacteria such as *S. bovis* (Park et al., 2020). For Metzger et al. (2018), the presence of bacterial DNA in feces and milk cannot confirm that bacterial DNA is entering the mammary gland from the intestine. DNA can also enter the mammary gland through the teat canal, coming from the animal's environment. The udder can be exposed to fecal bacteria when the cow is lying down, whereas the teat skin bacteria count is higher in cows with dirty legs and udders.

The sterilized milk from Dairy 2 (D2-S) presented as main: the *Enterobacteraceae* family with 140 sequences and *Acinetobacter haemolyticus* with 83 sequences, respectively 46.20% and 27.39% of the total sequences in the sample (303). *S. macedonicus* and *Pseudomonas azotoformans* appear in third place with only 8 sequences. Another twenty microorganisms appear with sequence numbers lower than eight.

By analyzing harmful microorganisms found in refrigerated raw milk from Dairy 1 and their prevalence in processed milks, it is possible to verify that UHT processing is inefficient for the elimination of species of the *Enterobacteriaceae* family and microorganisms such as *B. cereus group* and *A. dhakensis*. Regarding Dairy 2, processing proved to be inefficient in eliminating *Enterobacteriaceae*, *A. haemolyticus* and *S. equinus*.

Understanding the bovine milk microbiota lacks parameters established by current legislation. Thus, the data observed in the studies become difficult to interpret. Another important aspect is that the microorganisms present in the samples may come from bacterial contamination of collection utensils or laboratory materials. In addition, the DNA present in the samples may originate from inactive microorganisms, wholly or partially.

CONCLUSION

Samples from dairy products from Taquari Valley demonstrate a decrease in the number of microorganisms from refrigerated raw milk to processed milk. The Dairy 2 UHT process demonstrated significantly better quality than the Dairy 1 UHT process. The samples showed considerable diversity even within the same type of milk. Lactic acid bacteria such as S*. macedonicus* were found in refrigerated and pasteurized raw milk and *S. thermophilus* was found in sterilized milk. In addition, psychrotrophic species such as *K. gibsonni* and *B. cereus group* form a large part of the microbiota in the three types of milk. However, harmful species such as *B. cereus group*, *A. dhakensis* and *A. haemolyticus* were found in processed milk from Dairy 1 and Dairy 2, demonstrating that the processing used in both dairy factories needs to improve their efficiency in eliminating these microorganisms. Moreover, the elimination of members of the *Enterobacteriaceae* family of *S. equinus* is also necessary.

AUTHORS' CONTRIBUTIONS

Conceptual Idea: Müller, T.; Rempel, C.; Maciel, M.J.; Methodology design: Müller, T.; Maciel, M.J.; Data collection: Müller, T., Data analysis and interpretation: Müller, T.; Rempel, C.; Maciel, M.J. and Writing and editing: Müller, T.; Rempel, C.; Maciel, M.J.

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3. DISCUSSÃO GERAL

Esta seção apresenta uma discussão acerca dos artigos produzidos na presente tese, expostos na seção desenvolvimento, relacionando-os com o objetivo geral e com os objetivos específicos da pesquisa, fazendo a relação entre os artigos produzidos.

Essa pesquisa foi desenvolvida em etapas, no primeiro momento foi realizada a pesquisa bibliográfica sobre os aspectos envolvidos na qualidade do leite produzido e a interferência dos parâmetros utilizados na verificação da qualidade do leite em sua produção, a fim de produzir um alimento que possa atender às necessidades nutricionais do consumidor. No segundo momento foram realizadas as análises da microbiota do leite cru refrigerado, pasteurizado e esterilizado das indústrias da região do Vale do Taquari e no terceiro momento realizaram-se as coletas das amostras de leite cru refrigerado nas propriedades produtoras de leite e novamente dos três tipos de leite das indústrias da região. Essas amostras foram utilizadas para a realização das análises de composição do leite, análises físico-químicas e análises microbiológicas preceituadas pela legislação e, adicionalmente, contagem de microrganismos mesófilos, contagem de microrganismos psicrotróficos e análise de coliformes totais e termotolerantes. A análise de dados ocorreu de forma qualitativa e quantitativa.

O artigo 2 apresenta os resultados obtidos por meio das análises realizadas no leite das propriedades produtoras de leite e das indústrias da região estudada, realizando um comparativo no tocante aos aspectos físico-químicos, microbiológicos e de composição do leite. O leite cru refrigerado das propriedades apresentou uma qualidade superior ao leite cru refrigerado das indústrias, quando se levando em

considerações os aspectos microbiológicos e CCS, principalmente. As indústrias apresentaram maiores quantidades de CCS, microrganismos psicrotróficos e coliformes totais e termotolerantes. Em relação aos parâmetros físico-químicos, apenas duas propriedades apresentaram acidez acima do estabelecido pela legislação. Todas as propriedades analisadas apresentaram a maior parte dos parâmetros de composição do leite (gordura, lactose, proteínas, ST ou EST, SNG ou ESD) dentro do estabelecido pela legislação. A exceção ocorre apenas em uma propriedade (propriedade 33), em que o leite apresentou excesso de gordura. A gordura do leite em questão ficou estimada em 10 g/100g de leite. A legislação não estabelece um valor máximo para os parâmetros de composição do leite. O aumento da gordura da amostra pode estar relacionado a uma homogeneização incorreta ou insuficiente do leite no resfriador.

A análise de CCS, estabelecida na legislação apenas para o leite cru refrigerado, foi o parâmetro com maior desconformidade, estando acima do permitido nas duas indústrias e em 18 das 33 propriedades analisadas nesse estudo. A CCS indica mastite nos animais, que pode se manifestar de forma subclínica, dificultando o seu diagnóstico e o tratamento do animal. O leite do caminhão-tanque, proveniente de várias propriedades acaba por ser mais propenso ao aumento na quantidade de células somáticas (NDAHETUYE *et al.,* 2020), o que explicaria a quantidade de CCS acima do determinado no leite dos caminhões-tanque das indústrias.

Apesar de acima do permitido em ambas as indústrias, a CCS do leite cru refrigerado da indústria 2 foi bem inferior à da indústria 1, demostrando uma qualidade ligeiramente superior. Além disso, a indústria 2 apresentou somente esse parâmetro, dentre todos os analisados, acima do estabelecido pela legislação.

O leite apresentou CBT em desconformidade com a legislação para três propriedades e a indústria 1. Essa mesma indústria apresentou acidez acima do permitido para os três tipos de leite (cru refrigerado, pasteurizado e esterilizado) e densidade acima do permitido para o leite pasteurizado, além de teste de alizarol positivo. A CBT elevada pode ser a responsável pelo teste de alizarol positivo e a acidez acima do permitido na indústria 1. Já em relação às propriedades, apesar de o leite ter apresentado CBT acima do permitido, apenas o leite de uma das três propriedades apresentou também alizarol positivo, confirmando a presença de

microrganismos. Além disso, em nenhuma destas propriedades o leite apresentou-se com acidez acima do estabelecido. Isso pode ser explicado pelo fato de o leite ainda não ter se tornado ácido pela ação das bactérias, ou que as bactérias presentes nas amostras não são do tipo ácido láticas, responsáveis pela fermentação da lactose e produção de ácido lático que causa o aumento da acidez do leite

Ao realizar um comparativo geral entre propriedades e indústrias é possível constatar que o leite das indústrias demonstrou maiores quantidades de CCS, CBT, psicrotróficos e coliformes totais e termotolerantes que as propriedades produtoras de leite. O leite cru refrigerado e pasteurizado da indústria 2 apresentou uma qualidade microbiológica e físico-química superior à do leite cru refrigerado e pasteurizado da indústria 1. O leite esterilizado não demonstrou crescimento de colônias de microrganismos mesófilos, psicrotróficos e foi negativo na análise de coliformes totais e termotolerantes, evidenciando um tratamento efetivo pelas indústrias.

O artigo 3 apresenta os resultados das análises dos parâmetros físico-químicos, microbiológicos e de composição do leite cru refrigerado e dos leites processados pelas indústrias, juntamente com a análise da microbiota do leite a nível de gênero.

Em relação à contagem de microrganismos psicrotróficos, esta mostrou-se acima do recomendado pelos autores da área nas duas indústrias analisadas. Não há um consenso exato, mas a literatura traz que, quantidades de psicrotróficos acima de 1,0 x 10^5 UFC/mL ou 100.000 UFC/mL (RIBEIRO JUNIOR *et al.,* 2018) podem comprometer a qualidade do leite produzido.

A quantidade de psicrotróficos foi superior à CBT, no leite cru refrigerado e à contagem de microrganismos mesófilos, no leite pasteurizado das duas indústrias, além de ser superior na indústria 1, em relação a quantidade encontrada na indústria 2. Preconiza-se que a quantidade de microrganismos psicrotróficos seja em torno de 10% da CBT. O fato de a quantidade de microrganismos psicrotróficos ser muito ser superior na indústria 1 do que na indústria 2, mostra que a refrigeração do leite nas propriedades e nos caminhões-tanque da indústria 2 tem sido mais eficiente. Conforme demostrado no artigo 2, a quantidade de psicrotróficos nas propriedades produtoras de leite foi muito inferior à encontrada nas indústrias do VT, fato que pode ser explicado por esses microrganismos se reproduzirem em temperatura de

refrigeração, aumentando sua quantidade exponencialmente com um tempo de refrigeração prolongado. Dessa forma o tempo de armazenamento do leite nos caminhões-tanque é um fator decisivo na quantidade de psicrotróficos.

Em relação as análises da microbiota do leite coletado nas indústrias, foram encontradas 51.401 sequências do reino *Bacteria*, divididos em três filos, 15 ordens e 21 famílias. Em relação aos gêneros, as análises apresentaram um total de 41, demonstrando, dessa forma, uma grande diversidade. Os principais gêneros encontrados foram em sua maior parte já descritos em outros trabalhos (RIBEIRO JUNIOR *et al.*, 2018; RUEGG, 2022; RYU *et al.,* 2022) que avaliaram a microbiota do leite. Porém, o que pode ser constatado é que a diversidade é muito grande, mesmo no mesmo tipo de leite analisado (cru refrigerado, pasteurizado e esterilizado).

Os gêneros encontrados nesse estudo foram de microrganismos psicrotolerantes, como *Kurthia*, *Acinetobacter*, *Viridibacillus,* microrganismos formadores de biofilmes, como *Pseudomonas, Bacillus,* microrganismos mastitogênicos como *Streptococcus* e microrganismos ácido láticos como *Lactococcus,* nos três tipos de leite analisados.

Além dos gêneros descritos acima, já esperados em amostras de leite bovino, gêneros de microrganismos considerados nocivos como *Escherichia, Citrobacter*, *Aeromonas* e *Enterobacter* foram encontrados no leite cru refrigerado, mas também no leite pasteurizado e no leite esterilizado das indústrias. As análises de sequenciamento genético possibilitam uma visão mais aprofundada da qualidade do leite e a presença desses gêneros demostra falhas de higienização, no caso do leite cru refrigerado e um beneficiamento não tão eficiente no caso dos leites processados, somente detectados por meio de métodos mais sensíveis que os métodos convencionais, preconizados na legislação.

Os artigos 4 e 5 apresentam os resultados da análise da microbiota do leite cru refrigerado, pasteurizado e esterilizado das indústrias do Vale do Taquari, realizado por meio do sequenciamento de alto rendimento. Esses dados foram trabalhados em nível de espécie, fazendo um apanhado das principais espécies existentes nos três tipos de leite (cru refrigerado, pasteurizado e esterilizado) no caso do artigo 4 e, um comparativo entre os três tipos de leite de cada indústria analisada, no caso do artigo 5.

O leite beneficiado pela indústria demonstrou a presença de microrganismos benéficos à saúde humana, como *S. thermophilus* e *S. macedonicus*. Esses microrganismos são utilizados na fabricação de produtos lácteos, sendo considerados microrganismos de culturas iniciais aos processos de fermentação. A presença desses microrganismos no leite das indústrias do Vale do Taquari se mostra de acordo com o esperado, além de serem importantes para a saúde do consumidor.

O sequenciamento demostrou ainda a presença de espécies psicrotróficas, como *K. gibsonni* e *B. cereus group* formam boa parte da microbiota nos três tipos de leite. De acordo com os dados apresentados nos artigos 2 e 3, foi observado nas análises microbiológicas, uma grande quantidade de microrganismos psicrotróficos no leite das indústrias, fato que vai de acordo com as espécies psicrotróficas encontradas na análise da microbiota.

Em relação à quantidade de total de sequências de microrganismos (51.401 sequências) encontradas nas amostras de leite das indústrias de laticínios do Vale do Taquari, ambas as indústrias demonstraram uma diminuição significativa na quantidade do leite cru refrigerado para o leite processado pela pasteurização ou esterilização (UHT). Essa diminuição é também demostrada nas análises microbiológicas convencionais, conforme os dados apresentados nos artigos 2 e 3. Apesar disso, o processo UHT da indústria 2 demonstrou uma eficiência significativamente superior ao processamento UHT da indústria 1, eliminando mais de 90% dos microrganismos.

As análises demostraram ainda que os processos, além de efetivos na diminuição da quantidade total de microrganismos, eliminam microrganismos que causam deterioração do leite ou problemas à saúde do consumidor, como *L. sphaericus*, *L garviae*, *A. hidrofila*, *E. cloacae* e *A. baumanni*. Porém, espécies de microrganismos considerados nocivos como *B. cereus group, A. dhakensis, E. bacterium* e *A. haemolyticus* foram encontrados no leite beneficiado das indústrias 1 e 2, consoante ao já apresentado no artigo 3, à nível de gênero. Isso mostra que os processos térmicos não têm sido completamente eficientes na eliminação desses microrganismos e demonstrando ainda que, o processamento utilizado nas duas indústrias precisa melhorar a eficiência na eliminação desses microrganismos. Além

disso, a eliminação de membros da família *Enterobacteriaceae* e a espécie *S. equinus* também se faz necessária.

Ao realizar a comparação entre a microbiota existente nos três tipos de leite das duas indústrias analisadas, as amostras mostraram considerável diversidade mesmo dentre o mesmo tipo de leite, mostrando que espécies encontradas nos leites da indústria 1 não estavam presentes no mesmo tipo de leite da indústria 2 e vice-versa. Essa diversidade é grande a nível de gênero, conforme já demostrado no artigo 3, mas aumenta consideravelmente quando analisada à nível de espécie.

O sequenciamento de alto rendimento não possui parâmetros estabelecidos na legislação vigente, tornando difícil a interpretação dos resultados. A análise genética demonstrou que o leite UHT apresenta sequências de microrganismos, ainda que com quantidades muito diferentes entre as duas indústrias, sendo muito superior na indústria 1. A análise microbiológica convencional não evidenciou a formação de colônias nesse tipo de leite, porém, o sequenciamento é um método mais sensível. Ainda, é importante salientar que as sequências observadas por meio das análises moleculares podem pertencer a microrganismos inativos. Outra possibilidade é que essas sequências sejam provenientes de contaminação externa, por meio dos materiais de laboratório ou do próprio ambiente e utensílios durante a coleta das amostras.

A delimitação de diretrizes e parâmetros para a análise da microbiota do leite é uma perspectiva futura. As análises da microbiota podem ser agregadas às análises convencionais, já realizadas periodicamente em toda a cadeia leiteira. Isso pode ser de grande utilidade para um diagnóstico mais preciso do leite produzido, desde as propriedades produtoras de leite até a finalização do processo realizado na indústria. A partir do conhecimento da microbiota do leite é possível identificar especificamente os microrganismos causadores de problemas, como é o caso da mastite, que provoca o aumento da CCS e alterações na bioquímica e na composição do leite prejudicando consideravelmente a sua qualidade. Além disso, há a possibilidade de identificar microrganismos potenciais para a produção de diferentes tipos de lácteos, que podem vir a ser desenvolvidos e comercializados.

4. CONSIDERAÇÕES FINAIS

Esta pesquisa evidenciou a qualidade do leite produzido na região do Vale do Taquari – RS, analisando desde as propriedades produtoras de leite até o processamento do leite nas indústrias de laticínios da região. Os aspectos físico-químicos, bioquímicos e microbiológicos do leite mostraram que as propriedades produtoras de leite da região se encontram de acordo com o que preceitua a legislação. Em relação às indústrias, a indústria 2 apresenta uma qualidade do leite ligeiramente superior à indústria 1, porém, grande parte dos parâmetros analisados, principalmente os de composição do leite como lactose, proteínas, gordura, ST e SNG estão de acordo com a legislação em ambas as indústrias.

As análises microbiológicas adicionais como a contagem de microrganismos psicrotróficos e a análise de coliformes totais e termotolerantes realizados nos três tipos de leite analisados, cru refrigerado, pasteurizado e esterilizado, de indústrias e propriedades e a contagem de microrganismos mesófilos realizada no leite pasteurizado e esterilizado das indústrias são úteis na avaliação mais precisa da qualidade do leite produzido. Essas análises adicionais demostraram que os aspectos como a quantidade de psicrotróficos, não determinados na legislação e, por isso, não realizados periodicamente nas propriedades e laticínios, elencam aspectos importantes sobre o leite. As indústrias demostraram contagem de microrganismos muito mais altos do que os encontrados nas propriedades e, dessa forma, a questão da refrigeração adequada e prolongada o menor tempo possível precisa ser observado para a produção de um leite de qualidade.

O sequenciamento de alto rendimento, utilizado na análise da microbiota do leite produzido, demostrou uma diversidade microbiológica muito grande, com

aspectos a serem explorados e situações a serem melhoradas. A indústria 1 apresenta uma quantidade de sequências de microrganismos muito maior à observada na indústria 2, porém ambas apresentam grande diversidades de gêneros e espécies. Mais estudos são necessários para compreender de forma mais elaborada e efetiva a microbiota do leite produzido. Dessa forma, a metagenômica e a análise da microbiota podem ser grandes aliadas na produção de um produto de qualidade na região. Além disso, para facilitar esse processo, espera-se que diretrizes sejam produzidas, a fim de melhorar a interpretação desses dados.

A CCS foi o parâmetro que apresentou maior desconformidade, tanto nas indústrias como nas propriedades e a análise genética demostrou a presença de gêneros e espécies mastitogênicas. A realização de mais estudos com sequenciamento genético, com amostras de leite dos caminhões-tanque das indústrias, amostras *in loco* dos resfriadores das propriedades produtoras de leite, além de amostras coletadas individualmente nos animais são importantes para um diagnóstico mais preciso e específico. Assim, é possível rastrear a origem do problema, abrindo a possibilidade do tratamento efetivo e precoce dos animais com mastite subclínica.

A tese inicial, de que a qualidade do leite bovino produzido no VT atende aos parâmetros estabelecidos pela legislação vigente, porém pode ser melhorada por meio de análises que avaliem outros parâmetros não determinados pela legislação, como é o caso da contagem de microrganismos psicrotróficos, análise de coliformes totais e termotolerantes e sequenciamento genético, foi comprovada. As limitações do estudo ocorreram no fato de as coletas ocorreram em uma única época do ano e de que algumas propriedades não enviam o leite produzido para uma das duas indústrias analisadas neste estudo. Além disso, as coletas das amostras nas propriedades e indústrias ficaram condicionadas à aceitação dos participantes da pesquisa.

Em suma, a região do Vale do Taquari, que utiliza a pecuária leiteira como uma de suas principais atividades econômicas, possui um grande potencial a ser explorado. Por meio disso, é possível melhorar ainda mais a qualidade do leite e produtos lácteos produzidos na região, trazendo melhorias à saúde do consumidor e à qualidade de vida dos produtores.

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