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Microbiological quality and cultivable bacterial community of fresh and ripened Minas cheeses made from raw and pasteurised milk

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ABSTRACT

The microbiological and physicochemical characteristics of 56 samples of Brazilian fresh (Minas frescal cheese, MFC) and ripened (Minas padrão cheese, MPC; Minas artisanal cheese, MAC) cheeses produced from pasteurised (MFC and MPC) or raw milk (MAC) were evaluated. Significant differences were observed between samples, with a positive emphasis on microbiological quality for MPC. MALDI-TOF MS identified a diverse bacterial community from the 808 colonies isolated from different culture media. *Staphylococcus aureus* (34.32%) and *Escherichia coli* (35.59%) were the most isolated species. The *eae* gene, which confers virulence on *E. coli* strains, was observed in 23 samples (41.07%), and in 21.05%, 27.78%, and 73.68% of MFC, MPC and MAC samples, respectively. There was no correlation between the evaluated indicators and the presence of *eae*-positive cheese samples. The choice of the ideal microbiological indicator must be specific, considering the physicochemical characteristics and the raw milk of the different types of cheese.

1. Introduction

Cheeses are the main Brazilian dairy product, representing a large part of the national market (Araújo, Camargo, Carvalho, & Nero, 2020). Minas cheeses are traditional cheeses, typically Brazilian, named according to their characteristics and production method (Araújo et al., 2020; Brasil, 2002). Minas frescal cheese (MFC) is a white soft cheese, with 25.0 to 44.9% fat content and moisture content equal to or higher than 55.0%, produced from pasteurised milk (73–75 °C/15–20 s) or subjected to equivalent treatment (Brasil, 1997). Minas padrão cheese (MPC) is a cheese produced with pasteurised milk, matured for at least 20 days at 10–12 °C, with a creamy white to slightly yellow, semi-hard to a soft consistency, fat content between 42 and 57% and moisture content between 36 and 45.9%, with a culture of lactic acid bacteria as an ingredient (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) (Brasil, 2020; Furtado & Lourenço Neto, 1994). Fig. 1 illustrates the MFC and MPC production flowchart (Furtado & Lourenço Neto, 1994).

Changes in eating and consumption habits have led the Brazilian population to considerably increase the consumption of artisanal cheeses (Matera et al., 2018). Artisanal cheeses are made by specific non-standard protocols, using traditional methods, adding regional characteristics (Brasil, 2019). The Minas artisanal cheese (MAC) is a ripened cheese with firm consistency, colour and flavour typical of the region and uniform paste, elaborated on the property where the milk originated, from freshly milked raw milk (Brasil, 2002). MAC is produced from a natural starter culture, called pingo, obtained from the production of cheese the day before. Pingo transfers the microbiota from raw milk to cheeses, giving the identity of artisanal cheeses from each region, manifested in the aroma, flavour and acidity of the final products (Kamimura et al., 2019). MAC maturation time varies according to the region (Minas Gerais, 2021). The artisanal characteristic of cheese is also related to variations in the production flowchart. Fig. 1 provides the basic production flowchart for MACs (IMA, 2021; Monteiro & Matta, 2018).

Cheese is an ideal substrate for the development of spoilage and pathogenic microorganisms, with emphasis on *Listeria*

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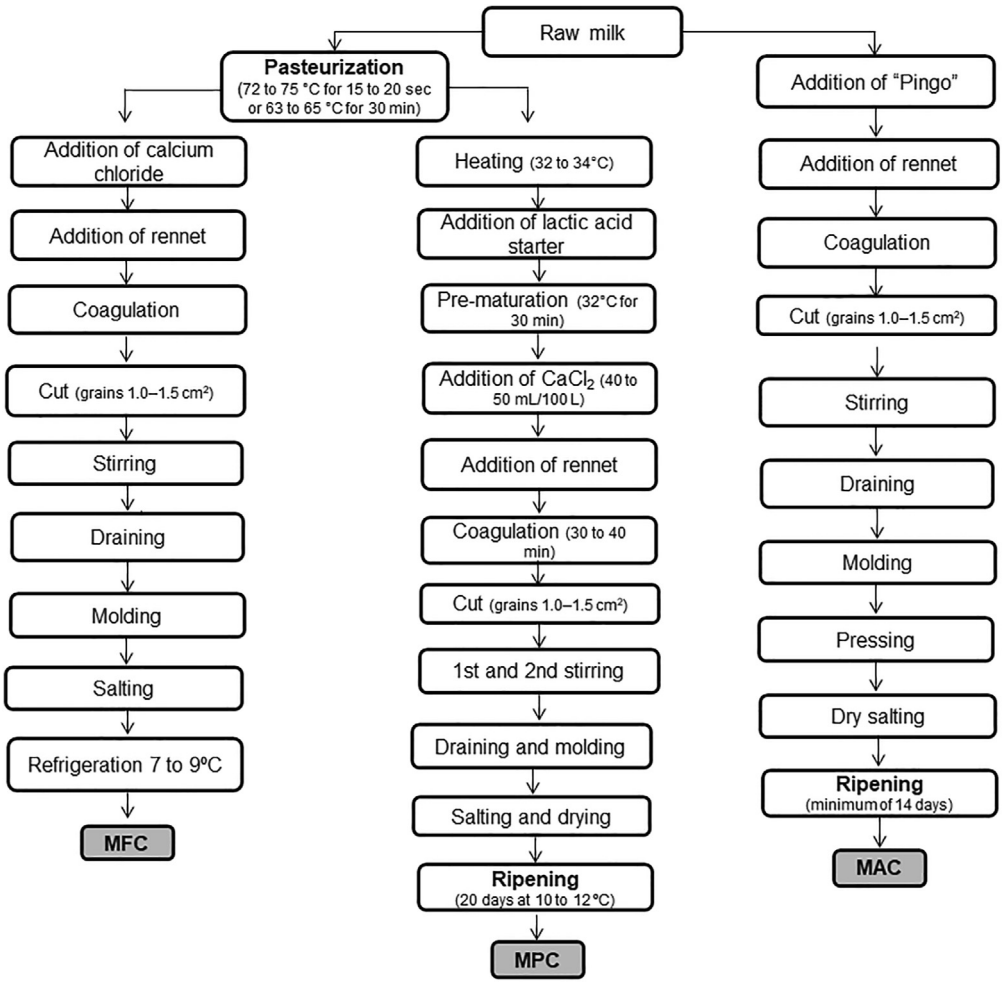

Fig. 1. Processing flowchart of Minas frescal cheese (MFC), Minas padrão cheese (MPC) (Furtado & Lourenço Neto, 1994) and Minas artisanal cheese (MAC) (IMA, 2021; Monteiro & Matta, 2018).

monocytogenes, *Staphylococcus aureus*, *Salmonella* spp. and diarrheagenic *Escherichia coli* (DEC), including enteropathogenic *E. coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC) (Farrokh et al., 2013). Intimate adhesion and destruction of intestinal cell microvilli are one of the main virulence factors of EPEC and STEC. This damage is caused by the production of a set of proteins, including intimin, which is encoded by the *eae* gene. The presence of the *eae* gene may be a predictor of potentially pathogenic *E. coli*.

The quality and safety of cheeses are influenced by the milk of origin, the process and hygienic conditions of manufacture, in addition to storage (Imran et al., 2019). The evaluation of different bacteria or bacterial groups has been a strategy to indicate the microbiological quality and safety of cheeses, providing information on the deterioration, the presence of pathogens, the possible source of contamination, as well as hygienic processing conditions (Andretta et al., 2019; Hervert, Alles, Martin, Boor, & Wiedmann, 2016).

European legislation establishes the count of *E. coli* and coagulase-positive staphylococci (CoPS), with different microbiological limits for fresh and ripened cheeses, produced with raw or pasteurised milk, in addition to the detection of *Salmonella* spp. and *L. monocytogenes* (European Commission, 2005, 2007). Brazilian legislation establishes, as microbiological criterion, the count of coliform total, coliform thermotolerant and CoPS, with different microbiological limits for cheeses classified according to their moisture content, not differentiating as to the raw material (raw or pasteurised milk), in addition to the detection of *Salmonella* spp and *L. monocytogenes* (Brasil, 1996).

The study of the cultivable bacterial community of cheese is of interest to the industry and government agencies, since the microbiological quality and safety of cheeses are investigated from cultivable microorganisms. Given the above, this study evaluated three different types of cheeses (MFC, MPC and MAC) regarding (i) their physicochemical characteristics, (ii) their microbiological quality and safety, (iii) the cultivable bacterial community; and (iv) the presence of the *eae* gene. In doing so, it was intended for this study to provide a set of data that contributes to the risk assessment and establishment of guidance microbiological criteria, considering the intrinsic, extrinsic and production differences of the types of cheese, resulting in the reduction of the risk for the consumer and the producer.

2. Materials and methods

2.1. Sample collection

Three types of Minas cheese (n = 56), produced from raw or pasteurised milk, ripened or not, MFC (n = 19; from seven different industries), MPC (n = 18; from six different industries) and MAC (n = 19; from 19 different producers) (Table 1) were randomly

Table 1
Minas cheese samples.[a]

Cheese	Pasteurised milk	Ripened	Number of samples	Number of industry/producers
MFC	Yes	No	19	7
MPC	Yes	Yes	18	6
MAC	No	Yes	19	19
Total			56	32

[a] Abbreviations are: MFC, Minas frescal cheese; MPC, Minas padrão cheese; MAC, Minas artisanal cheese.

collected in different establishments located in the state of Rio de Janeiro and Minas Gerais, from July 2018 to June 2019.

2.2. Physicochemical analysis

A total of 32 cheese samples (MFC, n = 7; MPC, n = 6; MAC, n = 19), one from each industry/producer, were evaluated for fat, moisture, protein, ash, fat in dry matter (FDM), water activity (Aw) and pH, according to the analytical standards previously described by Instituto Adolfo Lutz (IAL, 2008).

2.3. Microbiological analysis

Each cheese sample was fractionated into small pieces and 25 g were homogenised in 225 mL of three different broths: buffered peptone water (buffered peptone water; BPW; KASVI), modified tryptone soy bile novobiocin broth (mTSB; NEOGEN) and enrichment broth of Listeria (LEB; Kasvi, Brazil) in a Stomacher-type homogeniser (SP LABOR) for 2 min. From the sample diluted in BPW (10^{-1}), serial dilutions were performed in saline solution at 0.85% (w/v). Then microbiological analyses were performed.

The microbiological quality was performed by counting of total aerobic bacteria (TAB) (ISO 4833: 2; ISO, 2013), *Enterobacteriaceae* (EB) (ISO 21528-2; ISO, 2004), coliforms 45 °C (C45), *E. coli* (EC) (Kornacki, Gurtler, & Stawick, 2015), coagulase positive staphylococci (CoPS) (Bennet, Hait, & Tallent, 2015) and detection of *Salmonella* spp. (Andrews et al., 2018) and *L. monocytogenes* (Hitchins, Jinneman, & Chen, 2017).

Two to three different colonies, isolated from each culture medium aerobic plate count (APC) agar, violet red bile glucose (VRBG) agar, eosin-methylene blue (EMB) agar, Baird-Parker (BP) agar (all from Kasvi), xylose lysine deoxycholate (XLD) agar, hektoen enteric (HE) agar, bismuth sulphite (BS) agar (all from Plastlabor, Brazil) and polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol (PALCAM; Kasvi) agar were coded, their morphological characteristics, on the medium of origin, were annotated and then they were seeded in trypticase soy broth (TSB; Plastlabor) and incubated for 18 h at 35 °C. Afterwards, culture in TSB was seeded onto trypticase soy agar (TSA; Kasvi) and subjected to identification by matrix-assisted time-of-flight/laser-assisted desorption mass spectrometry (MALDI-TOF MS) (Rodrigues et al., 2017). In addition, 0.8 mL of the TSB culture was homogenised with 0.2 mL of glycerol and stored at −20 °C. The website www.namesforlife.com/ was consulted for the taxonomic classifications.

2.4. Detection of eae gene

Cheese samples in mTSB were seeded on cysteine lactose electrolyte deficiency agar (CLED; Kasvi) and incubated at 35 °C for 18−25 h. The polymicrobial culture grown in CLED was subjected to DNA extraction by thermal lysis (100 °C 10 min^{-1}), and investigated for the presence of the *eae* gene employing the polymerase chain reaction (PCR), as described by China, Pirson, and Mainil (1996).

2.5. Statistical analysis

Descriptive analysis was performed using the Microsoft Excel and the inferential analysis used Statistical Package for the Social Sciences (IBM SPSS, USA) software, version 18. Comparison of the three types of cheese, regarding the data on the variation of the indicators, was made using the Kruskal-Wallis non-parametric test and Mann-Whitney test was used for multiple comparisons. Also, EB, C45 and EC were compared as classifiers for sample adequacy using McNemar test. One-sample chi-square test was used to compare proportion of positive samples for each bacterial genus or species observed within MPC, MFC and MAC. An overall significance level of 5% and Bonferroni's correction for multiple comparisons, where adopted. Pearson's linear correlation coefficient was used to assess the existence of a linear relationship between the quantitative variables studied (logarithm of the counts).

3. Results and discussion

3.1. Physicochemical characteristics

The moisture was significantly higher in the MFC than in the MPC ($p = 0.001$) and the MAC ($p = 0.0001$) (Table 2). On the other hand, the fat content was significantly lower in the MFC than in the MPC ($p = 0.005$) and in the MAC ($p = 0.0001$) (Table 2). The moisture and fat content are related to the sensory characteristics of cheeses, influencing their shelf life (McSweeney, Fox, Cotter, & Everett, 2017).

No MFC, three (50%) MPC and 14 (73.68%) MAC showed the identity pattern established by Brazilian legislation (Brasil, 1997, 2002, 2020) (Supplementary material Table S1). Non-compliance with the identity standard generates uncertainties regarding the minimum quality characteristics of the product, which can lead to economic losses for the dairy industry. This can be controlled by standardising manufacturing processes (McSweeney et al., 2017). MAC are medium moisture cheeses (up to 45.9%; Brasil, 2002), produced by methods that value the historical and cultural tradition of the region, using specific protocols for each type and variety (Brasil, 2019). The high moisture content, in combination with the use of unpasteurised milk, can favour the development of spoilage and/or pathogenic microorganisms in artisanal cheeses.

The protein content was significantly lower in the MFC than in the MPC ($p = 0.008$) and MAC ($p = 0.0001$) (Table 2). The ripening process reduces the moisture content and, consequently, increases the fat and protein content (McSweeney et al., 2017). A negative correlation was observed between the moisture and fat content ($r = -0.74$; $p = 0.001$) and moisture and protein content ($r = -0.60$; $p < 0.001$). Taking into consideration the FDM content per 100 g of product, they can be classified, according to the legislation, as skimmed (<10%), low fat (10 to 24.9%), medium fat (25 to 44.9%), high fat (45 to 59.9%), or very high fat (≥60%) (Brasil, 1996). All the cheeses evaluated in this study were classified as high fat (46.2 to 48.2%) (Table 2).

The ash content and water activity of the cheeses are described in Table 2. Ash is related to the texture of cheeses, especially

Table 2
Mean (± standard deviation) of the fat content, moisture, protein, ash, water activity, fat in dry matter and pH of the Minas frescal cheese samples.[a]

Cheese	Fat (%)	Moisture (%)	Protein (%)	Ash (%)	FDM (%)	Aw	pH
MFC (n = 7)	20.57[a] ± 2.7	55.49[a] ± 3.1	19.67[a] ± 3.7	2.98[a] ± 1.1	46.23[a] ± 5.0	0.94[a] ± 0.0	5.7[a] ± 0.2
MPC (n = 6)	26.72[b] ± 2.0	44.48[b] ± 4.0	24.46[b] ± 2.0	3.53[a] ± 0.4	48.24[a] ± 3.8	0.93[a] ± 0.0	5.4[b] ± 0.1
MAC (n = 19)	26.26[b] ± 1.8	41.18[b] ± 5.4	27.33[b] ± 3.1	3.73[a] ± 0.7	46.40[a] ± 5.6	0.92[a] ± 0.0	5.2[c] ± 0.1

[a] Abbreviations are: Aw, water activity, FDM, fat in dry matter; MFC, Minas frescal cheese; MPC, Minas padrão cheese; MAC, Minas artisanal cheese. In each column, different letters indicate significance (p <0.017).

calcium, which acts as an important binding element (Matera et al., 2018). Although water activity is positively related to moisture (Andretta et al., 2019), the correlation between these two factors in cheese samples was weak (r = 0.46; p = 0.008).

Cheese samples were classified as low acidity product (pH 4.9 to 6.0) (Supplementary material Table S1), with significantly higher pH in MFC than in MPC (p = 0.001) and in MAC (p <0.0001). The pH was also significantly different between MPC and MAC (p <0.0001) (Table 2). The conversion of lactose into lactic acid reduces the pH of cheeses (McSweeney et al., 2017), the introduction of fermented whey originating from previous production (pingo) may be responsible for the lower pH of MAC (Bemfeito, Rodrigues, Silva, & Abreu, 2016).

3.2. Microbiological quality assessment

TAB is not an indicator to assess the microbiological quality of cheeses (Brasil, 1996, 2022; European Commission, 2005); however, spoilage processes can begin in foods with TAB counts greater than 6 log colony forming unit (cfu) g^{-1} (Jay, Loessner, & Golden, 2005). All MFC samples were classified as unsatisfactory (Table 3), as they had a TAB count above the microbiological limit established in this study (6 log cfu g^{-1}) (Supplementary material Table S2). The physicochemical characteristics of MFC, mainly moisture content and pH (Table 2, Supplementary material Table S1), favour microbial development. High TAB counts in MFC samples may be related to pasteurisation failures, poor hygiene during processing and/or improper storage temperature.

The TAB count is simple and inexpensive and does not require complex and differential culture medium. However, in ripened cheeses, as MPC and MAC, where the microbiota is desirable (Kamimura et al., 2019), the TAB count may have dubious interpretation. Indicators with doubtful interpretation significance should not be included as a microbiological criterion for the assessment of hygiene practices and health risks (Codex Alimentarius, 2020). Therefore, TAB cannot be used as a microbiological indicator of ripened cheeses. On the other hand, in fresh cheeses, the TAB count can be a good general indicator of microbiological quality.

Based on the literature (Imran et al., 2019), this study adopted the maximum limit of 3 log cfu EB g^{-1}. Thus, no MFC, 16 MPC (88.89%) and 6 MAC (31.58%) samples were classified as satisfactory (Table 3; Supplementary material Table S2), a significantly higher proportion of satisfactory MPC than MFC (p <0.0001) and MAC (p = 0.0006) samples (Table 3). The combination of pasteurisation and ripening may have been responsible for the low EB count in the MPC. EB in MFC may indicate pasteurisation failures, after pasteurisation contamination, and/or inadequate processing hygiene. Failures in hygiene practices may be related to high EB counts in the MAC. However, it is important to highlight that *Enterobacteriaceae* are commonly isolated from raw milk (Hervert et al., 2016), so the evaluation of EB in this type of cheese should be carefully assessed.

EB is an important indicator of microbiological quality for pasteurised milk (Hervert et al., 2016; Imran et al., 2019), but not for cheeses (Brasil, 1996, 2020, 2022; European Commission, 2005). However, some studies demonstrate the importance of EB counting in assessing the microbiological quality of dairy products (Hervert et al., 2016; Imran et al., 2019). The EB count is less direct than the TAB count, requiring a selective and differential culture medium.

According to the microbiological limits (Brasil, 2001), 18 (94.74%) MFC samples and all MPC and MAC samples were classified as satisfactory for C45 (Table 3; Supplementary material Table S2). However, the use of coliforms as a hygiene or pathogenic indicator for dairy products has been widely questioned (Hervert et al., 2016), no longer included in the latest legislation (Brasil, 2022; European Commission, 2005).

No cheese sample had a detectable level of EC, expressed as <0.5 log most probable number (MPN) g^{-1} (Supplementary material Table S2), being classified as satisfactory (Brasil, 2022) (Table 3; Supplementary material Table S2).

The number of satisfactory cheese samples for EB was significantly lower than for C45 (p <0.0001) and EC (p <0.0001) (Table 3). This is perhaps to be expected, since other gram-negative bacteria, in addition to those included in the coliform group, are part of this

Table 3
Number of samples (percentages in parentheses) of cheese classified as microbiologically satisfactory or unsatisfactory.[a]

Microorganism	MFC		MPC		MAC		All cheeses	
	S	UNS	S	UNS	S	UNS	S	UNS
TAB	0	19 (100)	NA	NA	NA	NA	0	19 (100)
EB	0[a]	19 (100)	16[b] (88.89)	2 (11.11)	6[a] (31.58)	13 (68.42)	22[a] (39.29)	34 (60.71)
C45	18[a] (94.74)	1 (5.26)	18[a] (100)	0	19[a] (100)	0	55[b] (98.21)	1 (1.79)
EC	19[a] (100)	0	18[a] (100)	0	19[a] (100)	0	56[b] (100)	0
CoPS	8[a] (42.11)	11 (57.89)	16[b] (88.89)	2 (11.11)	6[a] (31.58)	13 (68.42)	30 (53.57)	26 (46.43)
Salmonella spp.	19[a] (100)	0	18[a] (100)	0	19[a] (100)	0	56 (100)	0
L. monocytogenes	19[a] (100)	0	18[a] (100)	0	19[a] (100)	0	56 (100)	0
Microbiological quality	0[a]	19 (100)	14[b] (77.78)	4 (22.22)	3[a] (15.79)	16 (84.21)	17 (30.36)	39 (69.64)
Gene *eae*	Absence	Presence	Absence	Presence	Absence	Presence	Absence	Presence
	15[a] (78.95)	4 (21.05)	13[a] (72.22)	5 (27.78)	5[b] (26,32)	14 (73.68)	33 (58.93)	23 (41.07)

[a] Abbreviations are: MFC, Minas frescal cheese; MPC, Minas padrão cheese; MAC, Minas artisanal cheese; S, satisfactory; UNS unsatisfactory; TAB, total aerobic bacteria; EB, *Enterobacteriaceae*; C45, thermotolerant coliform, EC, *Escherichia coli*; CoPS, coagulase positive staphylococci; NA, not applicable. Microbiological limit: TAB (this study), EB (this study), C45 (Brasil, 2001), EC, CoPS, *Salmonella* spp., *L. monocytogenes* (Brasil, 1996). In each row of the MFC, MPC and MAC columns, different lowercase letters indicates significance (p <0.017). In the All cheeses column, different lowercase letters indicates significance (p <0.017), for the microorganisms EB, C45 and EC.

large EB group. C45 and EC are indicators that give a punctual response, with regard to process hygienic-sanitary quality and, specifically EC, faecal contamination (Hervert et al., 2016). On the other hand, EB can be suggested as a general indicator of microbiological contamination, with regard to the hygiene of fresh and ripened cheeses, produced with pasteurised or raw milk. In this aspect, the EB assessment would complement the EC assessment, reducing the consumer risk, which is the approval of a microbiologically unsatisfactory product (ICMSF, 2011). Other authors have also suggested EB as an indicator of hygienic practices for cheese (Hervert et al., 2016; Imran et al., 2019). However, it is important to consider that the regulation of many indicators, despite reducing consumer risk, can become excessive, requiring increased work, time and money (Kim et al., 2018), in addition to increasing producer risk, which is the disapproval of a microbiologically satisfactory product (ICMSF, 2011).

Intensive handling, characteristic of cheese production, and raw milk, mainly from subclinical mastitis, are important routes of contamination of cheeses by CoPS (Andretta et al., 2019). In view of this, CoPS are considered good indicators of handling hygiene and good manufacturing practices of cheese (Andretta et al., 2019). However, the presence of enterotoxigenic staphylococci does not guarantee the production of the toxin in the food, which requires specific conditions for production (Schwendimann et al., 2020). Thus, to assess the microbiological safety of staphylococcal intoxication, it is necessary to detect the staphylococcal enterotoxin produced in cheese (Brasil, 2022; European Commission, 2005). However, the detection of staphylococcal enterotoxin requires a more specific and expensive methodology, not accessible to many microbiology laboratories (Costanzo, Ceniti, Santoro, Clausi, & Casalinuovo, 2020).

The proportion of satisfactory MPC samples, in relation to the CoPS indicator, was significantly higher than for MFC ($p = 0.0051$) and MAC ($p = 0.0006$) (Table 3). The technological barriers to pasteurisation and ripening, together with good manufacturing practices, may have been the factors responsible for the control of CoPS in MPC samples. The high CoPS counts observed in the MPC and MAC samples (Supplementary material Table S2) indicate hygienic failures in production.

All cheese samples were negative for *Salmonella* spp. and *L. monocytogenes* (Table 3, Supplementary material Table S2). *Salmonella* spp. and *L. monocytogenes* are important foodborne pathogens associated with cheese consumption (Martínez et al., 2020). The absence of these bacteria in MFC may be associated with milk pasteurisation. In MPC, ripening, which results in low moisture, may have contributed to the absence of these pathogens and in MPC, the combination of pasteurisation and ripening may have influenced. *Salmonella* spp. and *L. monocytogenes* have been described in typical Brazilian cheeses (Martínez et al., 2020).

Cheese quality was determined from the evaluation of microbiological indicators. The number of satisfactory MPC samples was significantly higher than that of MFC ($p < 0.0001$) and MAC ($p = 0.0002$) (Table 3). Moisture content and pH may have influenced the microbiological quality of MFC and MPC samples. On the other hand, the physicochemical characteristics were not able to explain the difference observed, in terms of microbiological quality, between the samples of MPC and MAC. The microbiological quality of MAC samples may be related to the low microbiological quality of raw milk and/or failures in sample processing.

Although the cheese production stages, including transport and distribution, have not been evaluated, the results presented suggest that the combination of technological barriers, pasteurisation and ripening, is more efficient in microbial control, than when these technologies are used alone. During the pasteurisation and ripening of the MPC, pathogenic and spoilage microorganisms are eliminated (Kamimura et al., 2019).

Microbiological criteria, such as sampling plan, microorganism and microbiological limit, are adopted to assess the good practices employed in food production, and thus, ensure consumer safety (Codex Alimentarius, 2020). Different types of cheese vary in intrinsic and extrinsic characteristics, especially moisture content, making it a challenge to choose the ideal indicator. In addition, the choice of indicator must also consider the raw material (pasteurised or raw milk).

The evaluation of the microbiological quality of food must consider the risk of the producer and the risk of the consumer. Assessing broader groups of microorganisms increases the risk of the producer, however, decreases the risk of the consumer, the reverse can also occur (ICMSF, 2011).

3.3. Identification of isolates

Cheeses have a diverse microbiota, including yeasts, moulds and bacteria (Imran et al., 2019; Kim et al., 2018). The microbial community participates in the manufacture of different types of cheese, attributing sensory characteristics, as well as contributing to the control of pathogens and as indicator of the microbiological quality and safety of cheeses (Imran et al., 2019). A total of 808 bacterial colonies were identified by MALDI-TOF MS, 344 (42.57%) from MFC, 187 (23.15%) from MPC and 277 (34.28%) from MAC. Most isolates were Gram-positive bacteria (442; 54.70%), belonging almost exclusively to the Firmicutes phylum (440; 54.45%), while only two (0.25%) isolates (*Kocuria kristinae* and *Corynebacterium* spp.), isolated from MAC, belonged to the phylum Actinobacteria (Supplementary material Table S3). Gram-positive bacteria formed the majority of cultivable bacterial community of MFC (202; 58.72%) and MPC (103; 55.08%). On the other hand, in MAC, Gram-negative bacteria, represented by Proteobacteria, were slightly higher (140; 50.54%) than gram-positive bacteria (137; 49.46%). Gram-positive bacteria isolated from MFC were significantly higher ($p < 0.0001$) than Gram-negative bacteria. Gram-positive bacteria have been described as the most prevalent in cheese (Falardeau, Keeney, Trmčić, Kitts, & Wang, 2019; Imran et al., 2019; Kamimura et al., 2019). Gram-negative bacteria are important hygiene indicators and can also cause undesirable changes in cheese. However, beneficial actions such as pathogen control and desired sensory changes have been described in Gram-negative bacteria.

The vast majority of Firmicutes belonged to the order Bacillales (376/440; 85.45%), highlighting coagulase-negative *Staphylococcus* (CoNS) (202/440; 45.91%), represented by *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Staphylococcus sciuri* and *Staphylococcus warneri* and CoPS (151/440; 34.32%), represented by *S. aureus* (Supplementary material Table S3). CoPS and CoNS have been isolated from both, fresh and ripened cheeses (Gonzalez et al., 2017; Kim et al., 2018). In this study, the isolation of *S. aureus* (CoPS) was different and significantly higher in fresh cheese (MFC) (86/344; 25.00%) than in ripened cheeses (MPC and MAC) (65/464; 14.01%) ($p < 0.0001$) (Table 4).

Comparing cheese types, *S. aureus* isolation was significantly lower in MPC (9/187; 4.81%) ($p < 0.0001$) than in MFC (86/344; 25.00%) and MAC (56/277; 20.22%) (Supplementary material Table S3). This fact may be related to the high number of unsatisfactory MFC and MAC samples for the CoPS indicator (Table 3), mainly due to the handling of these products and the MFC does not go through the ripening process. It is noteworthy that, raw milk, used in MAC production, can be an important source of *S. aureus*. However, no significant difference was observed regarding the isolation of *S. aureus* between pasteurised (MFC and MPC) (95/531; 17.89%) and raw milk (MAC) (56/277; 20.22%) cheeses ($p = 0.4472$) (Table 4). In addition, the isolation of CoNS was different and significantly higher in MFC than in MPC ($p = 0.0259$) and MAC

Table 4
Number of important microorganisms isolated from cheese samples according to process and raw material.[a]

Microorganism	Process (%)		Raw material (%)	
	Fresh (n = 344)	Ripened (n = 464)	Pasteurised milk (n = 531)	Raw milk (n = 277)
Staphylococcus spp. (n = 353)	193 (56.10)[a]	160 (34.48)[b]	243 (45.76)[a]	110 (39.71)[a]
CoPS (n = 151)	86 (25.00)[a]	65 (14.01)[b]	95 (17.89)[a]	56 (20.22)[a]
CoNS (n = 202)	107 (31.10)[a]	95 (20.47)[b]	148 (27.87)[a]	54 (19.49)[b]
B. cereus (n = 15)	2 (0.58)[a]	13 (2.80)[b]	9 (1.69)[a]	6 (2.17)[a]
Lactobacillales (LAB) (n = 64)	7 (2.03)[a]	57 (12.28)[b]	48 (9.04)[a]	16 (5.78)[a]
E. coli (n = 121)	34 (9.88)[a]	87 (18.75)[b]	67 (12.62)[a]	54 (19.49)[b]

[a] Abbreviations are: CoPS, coagulase positive *Staphylococcus*; CoNS, coagulase negative *Staphylococcus*; LAB, lactic acid bacteria. In each row, different lowercase letters indicate significance (p <0.05), for process or raw material.

(p = 0.0012) (Supplementary material Table S3), also showing a significant difference between fresh (MFC) (107/344; 31.10%) and ripened (MPC and MAC) (95/464; 20.47%) cheeses (p = 0.0007) and between pasteurised (MFC and MPC) (148/531; 27.87%) and raw milk (MAC) (54/277; 19.49%) cheeses (p = 0.0101) (Table 4). Enterotoxin-producing *S. aureus* is most closely related to food poisoning (Langer et al., 2012). On the other hand, CoNS are also described as producing enterotoxins (Langer et al., 2012). In addition, *Staphylococcus* spp. resistant to multiple antimicrobials has been described as an important public health problem (Nunes, Souza, Pereira, Del Aguila, & Flosi, 2016). The difference in the number of *Staphylococcus* spp. (CoPS and CoNS) isolated was significant between fresh (MFC) and ripened (MPC and MAC) cheese samples (p <0.0001) (Table 4). The ripening process promotes physicochemical changes that will act directly on the intrinsic characteristics of the cheeses, which may have influenced the significantly lower number of *Staphylococcus* in the ripened cheese.

Bacillus cereus (15/440; 3.41%) was the third most prevalent Firmicutes (Supplementary material Table S3). Some authors have described the isolation of *B. cereus* in cheeses (Martínez et al., 2020). The difference in the number of *B. cereus* was significant for the three types of cheese evaluated (p = 0.032 2) (Supplementary material Table S3) and for the process (fresh or ripened cheese) (p = 0.0314) (Table 4). *B. cereus* are spore-forming bacteria and can survive thermal processes, additionally, they can produce emetic toxin in food and/or release diarrheal toxin in the human intestine (Hachiya et al., 2018).

Lactobacillales, represented by lactic acid bacteria (LAB), such as *Enterococcaceae*, *Lactobacillaceae* and *Streptococcaceae*, from starter and non-starter cultures, were isolated (Supplementary material Table S3). However, it is noteworthy that the number of LAB isolates (Supplementary material Table S3) was significantly higher (p <0.0001) in MPC than in MFC and MAC. Furthermore, the number of LAB isolates (Supplementary material Table S3) was also significantly higher (p = 0.0037) in MAC than in MFC. Anyway, the reduced number of contaminants may have favoured the development of LAB in the MPC. Comparing the processes, it was possible to observe a significant difference in the number of LAB isolated from fresh (MFC) (7; 2.03%) and ripened (MPC and MAC) (57; 12.28%) cheeses (p <0.0001) (Table 4). LAB is a group of nutritionally demanding bacteria (McSweeney et al., 2017), dominant in cheeses, mainly because they are involved in its production, consequently giving sensory characteristics (Falardeau et al., 2019). In this work, we do not use a specific culture medium for the isolation of LAB, which may justify the non-dominance of this group among the cheese samples evaluated, mainly among ripened cheeses, where LAB is part of the natural microbiota.

Enterobacteriales (340/366; 92.90%) was dominant among the Proteobacteria, with emphasis on the *Enterobacteriaceae* (293/340; 86.18%), followed by *Morganellaceae* (39/340; 11.47%) and *Hafniaceae* (8/340; 2.35%) (Supplementary material Table S3). *Enterobacteriaceae*, and other Enterobacteriales, such as *Hafnia alvei*, have been associated with gas production, consequently generating eyes in cheese (Arcuri, Sheikha, Rychlik, Piro-Métayer, & Monet, 2013).

Enterobacteriaceae are commonly isolated from cheese (Kamimura et al., 2019). The difference regarding *Enterobacteriaceae* in the three types of cheese (p = 0.7810) (Supplementary material Table S3), as well as in the process (fresh and ripened) (p = 0.7117) and raw material (pasteurised and raw milk) (p = 0.4887), was not significant (Table 4). *E. coli* (121/293; 41.30%) was the most prevalent *Enterobacteriaceae* (Supplementary material Table S3), with a different and significantly higher number of isolates in MPC (33/187; 17.65%; p = 0.0134) and MAC (54/277; 19.49%; p = 0.0008) than in the MFC (34/344; 9.88%) (Supplementary material Table S3). *E. coli* is part of the natural intestinal microbiota of humans and other animals. On the other hand, some strains are associated with intestinal and/or extra-intestinal infections (Gonzalez & Cerqueira, 2019). However, no cheese sample showed a detectable value of the EC indicator (Table 3, Supplementary material Table S2).

Undetectable *E. coli*, by conventional MPN test, may not always indicate the absence or low prevalence of this bacterium. The different culture media used, mainly trypticase soy agar and violet red bile glucose agar (data not shown), may have favoured the isolation of *E. coli*.

Several species of opportunistic *Enterobacteriaceae* were isolated, with emphasis on *Klebsiella pneumoniae* (35/293; 11.95%), with significantly greater isolation (p <0.0001) in MPC (23/187; 12.39%) than in MFC (4/344; 1.16%) and MAC (8/277; 2.89%); and *Enterobacter* spp. (32/293; 10.92%), with significantly greater isolation in MFC samples (25/344; 7.27%), than in MPC (2/187; 1.07%; p = 0.0014) and MAC (5/277; 1.81%; p = 0.0013) (Supplementary material Table S3).

H. alvei was isolated from pasteurised cheeses, while *Morganellaceae* were isolated from ripened cheeses (Supplementary material Table S3). *H. alvei* is a commensal microorganism widely distributed in nature (Imran et al., 2019), which, like *Morganella morganii*, *Proteus* spp. and *Providencia reuteri*, may eventually act as a human opportunistic pathogen (O'Hara, Brenner, & Michael, 2000).

Other Gammaproteobacteria, of the order Pseudomonadales (*Acinetobacter baumannii* and *Pseudomonas* spp.) were isolated from the three types of cheese (Supplementary material Table S3). *A. baumannii* and *Pseudomonas aeruginosa* may be involved in nosocomial infection (Imran et al., 2019).

Gram-negative opportunistic human pathogens, of the Alphaproteobacteria class, such as *Brevundimonas* spp., *Ochrobactrum intermedium* and *Alcaligenes faecalis* (Imran et al., 2019), were isolated (Supplementary material Table S3).

Actinobacteria was the least expressive phylum (2/808; 0.25%), with *K. kristinae* and *Corynebacterium* spp. isolated from MAC

(Supplementary material Table S3). Actinobacteria are generally found on animals' teats, being common in raw milk (Falardeau et al., 2019), which may justify the presence of these isolates in MAC.

The cheese production process, concerning fresh or ripened cheese, has a greater influence on the type and number of microorganisms in the product than the type of raw material (pasteurised or raw milk) (Aguiar et al., 2022).

The absence of *Salmonella* spp. and *L. monocytogenes* indicate that the cheese samples evaluated do not present a risk to consumer's health concerning these target pathogens. However, the isolation of potentially pathogenic bacteria, such as *S. aureus*, *B. cereus* and *E. coli* (Table 4), indicate the potential risk of this product to consumer health.

The classification of a bacterium as pathogenic or non-pathogenic depends, mainly, on the investigation of its virulence profile (Imran et al., 2019). Considering that cheese is a ready-to-eat food with access to a large part of the population, it is important to emphasise that future studies should be carried out to investigate the virulence potential of isolated bacteria, including the assessment of the antimicrobial resistance profile.

3.4. Detection of eae gene

The *eae* gene was present in 23 (41.07%) cheese samples, where the MAC *eae*-positive samples (14; 73.68%) were significantly greater than the MFC (4; 21.05%; $p = 0.0029$) and MPC (5; 27.78%; $p = 0.0086$) samples (Table 3). Although other virulence markers have been used to identify specific categories of DEC, the investigation of the *eae* gene is a great strategy for screening EPEC (typical and atypical) and STEC involved in serious diseases such as haemorrhagic colitis and haemolytic uremic syndrome (D'Auriac & Sirevåg, 2018). Animals, mainly bovines, are the main hosts/reservoirs of EPEC and STEC (Gonzalez & Cerqueira, 2019), being able to contaminate milk and, consequently, cheese. This may justify the higher prevalence of the *eae* gene in MAC, indicating the importance of heat treatment of milk to guarantee the safety of this product. However, good agricultural and manufacturing practices can ensure the quality and safety of raw milk cheeses. Humans can also contaminate milk and cheese with EPEC and STEC (Farrokh et al., 2013).

In Brazil, some studies have identified *eae*-positive *E. coli* in fresh and ripened cheeses (Parussolo et al., 2019). The presence of the *eae* gene in cheeses, mainly in MAC, indicates the potential risk of this food to the consumer's health, especially if STEC is present.

E. coli present individual diversities that classify them from commensal strains, inhabitants of the intestinal microbiota, to strains causing extra-intestinal infections (ExPEC) and intestinal infections (DEC) (Gonzalez & Cerqueira, 2019). The use of an indicator capable of informing the risk potential of a food for different categories of DEC would be of great advantage for microbiological assessments. In this study, no correlation was observed between the indicators evaluated and the presence of cheese samples carrying the *eae* gene. Therefore, the safety of the cheeses, regarding *E. coli eae*-positive, can only be evaluated from the research of the pathogenic bacteria.

4. Conclusion

Fresh and ripened cheeses differed in terms of physicochemical characteristics, and this was reflected in the microbiological quality, in the cultivable bacterial community and in the presence of the *eae* gene. Ripening exerted a greater influence on the satisfactory microbiological quality of the cheeses than the type of raw material used (pasteurised or raw milk). On the other hand, pasteurisation was related to the presence of the *eae* gene, where raw milk cheeses were highly contaminated with microorganisms carrying this virulence gene. Although the virulence profile and antimicrobial resistance were not investigated, the results presented demonstrate that the cheeses may pose a risk to consumer health, as well as to public health, with regard to potential isolated pathogens, in addition to *eae* gene identification. The combination of indicators is the best strategy to reduce the microbiological risk of cheeses and its choice must consider the intrinsic and extrinsic characteristics and the technological production of the cheese. The results presented in this study can help regulators and legislators to consider microbiological criteria for assessing the quality and safety of fresh and ripened cheeses produced with raw or pasteurised milk.

Author statement

Alice G. M. Gonzalez: Conceptualization. Cíntia B. Silva: Data curation. Ana Beatriz M. Fonseca: Formal analysis. Rossiane M. Souza and Alice G. M. Gonzalez: Funding acquisition. Cíntia B. Silva and Letícia M. Ferreira: Investigation. Cíntia B. Silva: Methodology. Alice G. M. Gonzalez: Project administration. Alice G. M. Gonzalez and Rossiane M. Souza: Resources. Alice G. M. Gonzalez, Rossiane M. Souza, Adriene R. Lima and Kátia G. L. Araújo: Supervision. Adriene R. Lima and Ana Beatriz M. Fonseca: Validation. Cíntia B. Silva: Visualization. Cíntia B. Silva: Original draft. Alice G. M. Gonzalez, Rossiane M. Souza, Adriene R. Lima and Ana Beatriz M. Fonseca: Review and editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2023.105662.

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