Microbiological quality of pasteurized human milk from a Milk Bank of São Paulo

Qualidade microbiológica do leite humano pasteurizado de um Banco de Leite Paulista

Calidad microbiológica de la leche humana pasteurizada de un banco de leche paulista

Claudete de Oliveira[1] https://orcid.org/0000-0002-7385-5371
Luís Carlos Lopes-Júnior[2] https://orcid.org/0000-0002-2424-6510
Cristina Paiva de Sousa[1] https://orcid.org/0000-0001-7151-5085

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Corresponding author
Claudete de Oliveira.
E-mail: claudete@ufscar.br

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Adriana Amorim Francisco
(https://orcid.org/0000-0003-4705-6987)
Escola Paulista de Enfermagem, Universidade Federal de São Paulo, São Paulo, SP, Brazil

Abstract

Objective: To assess the microbiological quality of pasteurized human milk from a Human Milk Bank in the State of São Paulo.

Methods: This is a descriptive study conducted with 29 pasteurized expressed human milk (PEHM) samples obtained between July 2015 and March 2016 by assessing titratable acidity records as well as quantifying heterotrophic microbiota (mesophiles, psychrophiles, thermophiles), total and thermotolerant coliforms, filamentous and yeast-like fungi and *Staphylococcus* spp. The physical-chemical parameters were assessed via hydrogen-pH potential, K-energy content and Dornic-ºD acidity. Descriptive and bivariate statistical analyzes were conducted.

Results: The presence of psychrophiles (17.24%), thermophiles (27.59%), mesophiles (55.17%), filamentous and yeast-like fungi (41.38%) and absence of *Staphylococcus* spp were evidenced in the sample. The presence of 82.76% of coliforms was detected in the presumptive test. In the confirmatory VB test, the presence of 54.16% of total coliforms was found and, in the EC test, we verified 33.33% of thermotolerant coliforms. The pH and K values did not show oscillations, whereas, in the expression of acidity between 3º and 15°D, microbial growth was detected. The mesophilic microorganism showed a positive correlation with the Dornic acidity variable (r=0.44; p=0.01).

Conclusion: Based on the microbiological quality assessment of the HMB samples discarded and considered unfit for consumption in the HMB, specifically regarding the microbiological indicators of hygiene conditions, it suggests that the infeasibility of the samples may be associated with good food handling practices.

Resumo

Objetivo: Avaliar a qualidade microbiológica do leite humano pasteurizado proveniente de um Banco de Leite Humano do Estado de São Paulo.

Métodos: Estudo descritivo conduzido com 29 amostras de leite humano ordenhado pasteurizado (LHOP) obtidas entre julho de 2015 a março de 2016 por meio da avaliação dos registros da acidez titulável bem como da quantificação da microbiota heterotrófica (mesófilos, psicrófilos, termófilos), coliformes totais e termotolerantes, fungos filamentosos e leveduriformes e *Staphylococcus* spp. Realizou-se a avaliação dos parâmetros físico-químicos por meio do potencial hidrogeniônico-pH, teor energético-K e acidez Dornic-ºD. Análises estatísticas descritivas e bivariadas foram conduzidas.

Resultados: Evidenciou-se nas amostras a presença de psicrófilos (17,24%), termófilos (27,59%), mesófilos (55,17%), fungos filamentosos e leveduriformes (41,38%) e ausência de *Staphylococcus* spp. Detectou-se a presença de 82,76% de coliformes no teste presuntivo. Já no teste confirmativo VB constatou-se a presença

[1]Universidade Federal de São Carlos, São Carlos, SP, Brazil.
[2]Universidade Federal do Espírito Santo, Vitória, ES, Brazil.
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de 54,16% de coliformes totais e no teste EC 33,33% de coliformes termotolerantes. Os valores de pH e de K não apresentaram oscilações, enquanto que, na expressão da acidez entre 3º a 15°D detectou-se crescimento microbiano. O microrganismo mesófilo, apresentou correlação positiva com variável da acidez Dornic (r=0.44;p=0.01).

Conclusão: A partir da avaliação da qualidade microbiológica das amostras de LHOP descartado e consideradas impróprias para consumo no referido BLH, especificamente com relação aos indicadores microbiológicos das condições de higiene, sugere que a inviabilidade das amostras possam estar associadas às boas práticas de manipulação do alimento.

Resumen

Objetivo: Evaluar la calidad microbiológica de la leche humana pasteurizada proveniente de un banco de leche humana del estado de São Paulo.

Métodos: Estudio descriptivo realizado con 29 muestras de leche humana ordeñada pasteurizada (LHOP) obtenidas entre julio de 2015 y marzo de 2016 por medio de la evaluación de los registros de acidez titulable, así como de la cuantificación de la microbiota heterótrofa (mesófilos, psicrófilos, termófilos), coliformes totales y termotolerantes, hongos filamentosos y levaduriformes y *Staphylococcus* spp. Se realizó la evaluación de los parámetros físico-químicos mediante el potencial de hidrógeno (pH), valor energético (K) y acidez Dornic-ºD. Se llevaron a cabo análisis descriptivos y bivariados.

Resultados: Se observó en las muestras la presencia de psicrófilos (17,24 %), termófilos (27,59 %), mesófilos (55,17 %), hongos filamentosos y levaduriformes (41,38 %) y ausencia de *Staphylococcus* spp. Se detectó la presencia del 82,76 % de coliformes en la prueba presuntiva. Por otro lado, en la prueba confirmativa VB se confirmó la presencia del 54,16 % de coliformes totales, y en la prueba EC se verificó el 33,33 % de coliformes termotolerantes. Los valores de pH y de K no presentaron oscilaciones, mientras que se detectó crecimiento microbiano en la expresión de la acidez entre 3 y 15°D. El microrganismo mesófilo presentó correlación positiva con variable de la acidez Dornic (r=0.44; p=0.01).

Conclusión: A partir de la evaluación de calidad microbiológica de las muestras de LHOP descartadas y consideradas inapropiadas para consumo en el BLH mencionado, especialmente respecto a los indicadores microbiológicos de las condiciones de higiene, se sugiere que la inviabilidad de las muestras pueda estar asociada con las buenas prácticas de manipulación del alimento.

Introduction

Scientific literature is quite consistent to assume that Human Milk (HM) as being the main food source in newborns and infants.[1–4] Among the benefits of this practice, there is an increase in survival, through the promotion of health and child development, in addition to the biological and psychosocial benefits for the development of babies.[1,5–7] In HM, in addition to antibodies, there are other factors with antimicrobial and immunomodulatory activity, such as enzymes, cytokines, components of the complement system, oligosaccharides, nucleotides, lipids and hormones, which contribute to the immunity and maturation of newborns' immune system.[8–11]

Scholars in the field of breastfeeding and HM point out that in situations of vulnerability including prematurity, recurrent hospitalizations of newborns in neonatal units, as well as mothers' diseases, or low milk production, can lead to difficulties in establishing and maintaining breastfeeding.[7,12,13] In these situations, the use of donated HM transposes itself as an efficient option for nutrition of newborns in special conditions[2,5] and a way of maintaining milk production by donor nursing mothers.[5,14] A recent systematic review that aimed to identify the activities of Human Milk Banks (HMB) showed that the respective actions developed in them have a positive impact on the promotion of maternal and child health, representing an important strategy to promote breastfeeding and support breastfeeding of babies who cannot suck directly on the breast.[15] The authors also pointed out that research on HMB as promoters of maternal and child health is scarce and more studies are needed to support public health strategies in favor of breastfeeding.[15]

Therefore, the existence and operationalization of HMB as a specialized service is crucial to trigger actions to promote, protect and support breastfeeding, as well as to carry out HM collection, processing, quality control and distribution activities.[16,17] In this sense, the process of quality of expressed human milk (EHM) ranges from milking to its administration and is a result of adequate hygienic sanitary conditions. Such process is defined through the joint assessment of multiple parameters including nutritional, immunological, chemical and microbiological characteristics, in order to provide verification of the final product safety.[16,18]

The steps involving the procedures for EHM collection show risks of contamination and, therefore, need to be carefully monitored.[16,19–23] It is the responsibility of HMB to supply innocuous, harmless EHM that fulfills its function as food, since pasteurization inactivates 95% of pathogens.[16,20–23]

Moreover, its quality is limited by inspection control in physical-chemical and microbiological testing until the final product is released, and sanitary hygienic conditions are the accepted parameter to enable its microbiological quality.[20–24]

The causes of elevation of microorganisms in the HM may be related to inadequate collection techniques, hygiene conditions of donors and utensils and the maintenance of milk outside the cold chain. The growth of bacteria in HM produces acidification and fermentation which can lead to a decrease in nutritional and immunological components due to the use of milk nutrients by the contaminating microbiota and a decrease in immunoprotective factors.[24–27]

Since HM is an excellent culture medium and has no obstacle to the microbial flora that is associated with the availability and quality of food, it is of paramount importance for maternal and child health that good practices in the handling process are ensured to guarantee its quality.[17,22,24–27] Given the theme relevance, added to maternal and child indefensibility in the consumption of HM distributed by HMB, is that this research is pertinent.

Although there are two previous studies, both addressed the management of the working process of assistance and human milk processing activities of HMB under investigation in this research with a focus on improving quality.[28,29] However, the assessment and microbiological quality of pasteurized human milk from the above-mentioned HMB are still unknown. Also, one of the differences of this study is that we assess the microbial growth in different types of temperature, allowing the association of temperature with the environment, storage, with the degradation of milk, among other parameters. In this context, the present study aimed to assess the microbiological quality of pasteurized HM from a Human Milk Bank in the State of São Paulo.

Methods

This is a descriptive study, carried out through the analysis and processing of EHM samples obtained in 2015/2016 at the HMB linked to *Irmandade da Santa Casa de Misericórdia de São Carlos*, São Paulo, Brazil. This study was approved by the Institutional Review Board of *Universidade Federal de São Carlos* - UFSCar, under Opinion 1.8088.916 of May 12, 2015, in line with the regulatory standards contained in Resolution 466/12 of the Brazilian National Health Council (*Conselho Nacional de Saúde*) regarding the conducting research with human beings.[30] Additionally, approval was obtained from the Internal Ethics Committee of *Irmandade da Santa Casa de Misericórdia de São Carlos* - an instance in which HMB is linked.

The sampling process was non-probabilistic and for convenience, covering all consecutive HM samples. Twenty-nine (29) samples were obtained by home collection and 01 sample was obtained by collection at HMB by donors registered at the HMB linked to *Irmandade da Santa Casa de Misericórdia de São Carlos* in the pre-established period of data collection of this study, which took place between July 2015 and March 2016. Thirty bottles were collected, containing 250 ml of EHM in each, qualified as inappropriate for consumption, with 01 sample of raw HMI that was discarded in the dirt phase and 29 bottles of pasteurized EHM, which were discarded in the assessment phase of titratable acidity expressed in Dornic degrees (ºD) and microbiological quality, as established in RDC 171.[20] It is noteworthy that the sample of raw EHM was discarded due to the absence of similar samples for comparisons. Moreover, we did not obtain or use EHM samples in the previous stages, analyzing packaging, dirtiness, color, and off-flavor. Thus, the final sample of this study consisted of 29 bottles of pasteurized HM. The samples were transported through the cold chain from the collection site at the HMB to the Laboratory of Microbiology and Biomolecules at UFSCar in isothermal containers, respecting the norms established by RDC 171,[20] stored in the freezer under a temperature of -3ºC for further processing microbiological analysis within 24 hours. Microbiological analysis was guided by the method recommended by the American Public Health Association (APHA).[31]

Analysis of the physical-chemical parameters of the potential of hydrogen (pH) of HM occurred

shortly after the defrost in a water bath at 40°C, being controlled so that the water temperature remained at up to 5°C. Subsequently, the flask was immersed, rising above the height of the level of HM and performing light shakes. After 5 minutes, HM was placed in a Becker (50ml), measuring the pH by immersing the electrode in HM for 1 minute to perform the reading. This procedure was repeated three times for each sample and we obtained the arithmetic mean for the pH value recorded for later statistical analysis. It should be noted that the physical-chemical parameters of energy content (K) as well as the results of the titratable acidity assessment[32] were carried out in the aforementioned HMB.

The detection of heterotrophic microorganisms started with the serial dilution of the HM sample in a peptide saline solution. Each sample was pipetted in a flame field in serial dilutions, with the initial dilution 10^0 consisting of 10ml of HM, followed by dilutions 10^{-1} and 10^{-2}. The identification of heterotrophic microorganisms was carried out according to the method previously described by APHA.[31] 1ml aliquots of the HM sample and its serial decimal dilutions were seeded in duplicates, by the inoculation technique in sterile petri dishes (90 x 15mm) containing 10 to 15 ml using the Plate Count culture medium (PCA – Himedia®). Surface inoculation with the aid of a Drigalski loop, allowed the plates to be incubated inverted at 37°C for 24h for the group of mesophiles; at 42.5°C for 24h for the group of thermophiles; at 7°C for 7 days for the group of psychrophiles. Colonies were counted and results were expressed in colony-forming units (CFU) per ml of HM.

For analysis of *Staphylococcus* spp, we proceeded according to the method proposed by APHA[31] with the use of the culture medium Agar Sal Mannitol (Kasvi®), in which the plates were incubated and inverted at 37°C for 24h to check for the presence of *Staphylococcus* spp colonies. The results were expressed in colony-forming units (CFU) per ml of HM.

For the quantification of filamentous and yeast-like fungi, we proceeded again with the method described by APHA.[31] Thus, we used the culture medium Agar Sabouraud Dextrose (Kasvi®), in which the plates were incubated and inverted at 37°C for 24h, to detect the presence of colonies of these fungi. The results were also expressed in colony forming units (CFU) per ml of HM.

The presence of total coliforms and thermotolerant coliforms were determined by the Most Probable Number (MPN) technique, according to the method described in a previous study.[33] The MPN test of total and thermotolerant coliforms was based on two tests, namely the presumptive and the confirmatory test for a series of 3 tubes. The presumptive test was performed using a 1ml aliquot of HM in a tube containing 9ml of Sodium Lauryl Sulfate Broth (Kasvi®) in serial dilutions of 10^{-1}, 10^{-2} and 10^{-3} succeeding the incubation at 37°C for 24h. The confirmatory test was initiated after the verification of the inoculation of the positive tubes through the formation of gas and/or turbidity of the medium. This phase consisted of transferring 1 ml of the serial and triplicate dilution to tubes containing 9 ml of Brilliant Green Bile Lactose (VB) 2% (BGBL - (Kasvi®), and to tubes containing 9 ml of EC Broth (Kasvi®), followed by incubation at 37°C and 42.5°C for 24h to 48h. The tubes with gas and/or turbidity, in both tests were recorded in order to quantify MPN/ml of HM.

For data analysis, they were initially digitized in Microsoft Excel™ spreadsheets, and, after coding, they were exported to the Statistical Package for Social Sciences program (SPSS Inc., Chicago, United States), version 20.0, to proceed with statistical, descriptive, and bivariate analysis. Ordinal and nominal qualitative variables were presented based on absolute and percentage numbers, via relative frequency distribution (%) and the discrete and continuous quantitative variables, through standard deviation (SD), median, minimum value (min) and maximum value (max). Then, we proceeded with bivariate statistical analyzes to investigate the correlation between the variables of the studied microbiota and °Dornic acidity using Pearson's correlation coefficient. For all analyzes, α=5%, α=0.20 and 95% Confidence Interval were set, with the significance level set at $p<0.05$.

Results

Titratable acidity

The average of results of assessment of titratable acidity that identifies the level of acidity expressed in Dornic degrees (ºD) of the EHM samples in HMB was 7.8ºD, standard deviation (SD)=2.29ºD (minimum value 3ºD, value maximum 15ºD). Moreover, 76.6% of EHM samples (n=23) had a titration degree ≥ 8ºD, considered unfit for consumption. Regarding the energy content (K), an average of 587 Kcal/l was observed in EHM samples of HMB, SD=77.4 Kcal/l. In addition, the pH mean of EHM samples analyzed was 6.8 (SD=0.22). Table 1 presents an overview of physical-chemical analysis of Dornic acidity, pH, K and the quantification of each microbial group.

Microbiological culture

Table 2 shows the quantification of coliforms in the presumptive test, confirmatory test VB and EC respectively. The absence of coliforms was observed

Table 1. Physical-chemical analysis and bacterial quantification of pasteurized expressed human milk

Sample Nº	Chemical-physical analysis			CFU/ml														MPN g/ml		
				Psychrophiles			Mesophiles			Thermophiles			Filamentous fungi			*Staphylococcus sp*			Coliforms		
	ºD	pH	K	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}	Presumptive	VB	EC
1	8	6.9	595.2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
2	8	6.9	584.0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
3	8	7.0	586.1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
4	8	6.7	586.1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	$23x10^0$	--	--
5	8	6.7	494.5	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	$9.2x10^0$	--	--
6	10	6.7	486.4	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	$23x10^0$	--	--
7	5	6.9	493.4	1.5	--	--	--	--	--	--	--	--	--	--	--	--	--	--	$240x10^0$	$21x10^0$	--
8	8	7.1	581.4	6	0.1	--	45	0.2	--	--	--	--	--	--	--	--	--	--	--	--	--
10	8	7.2	486.4	--	--	--	--	--	--	0.5	--	--	1.5	--	--	--	--	--	$21x10^0$	--	$21x10^0$
11	3	6.6	584.7	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	$21x10^0$	$21x10^0$	--
12	5	6.0	682.9	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	$3.6x10^0$	$3.6\ x10^0$	--
13	8	6.7	583.6	--	--	--	15	0.5	--	--	--	--	--	--	--	--	--	--	$3.6x10^0$	$3.6\ x10^0$	--
14	9	6.7	593.6	5	--	--	20	0.25	0.05	--	--	--	16	--	--	--	--	--	$3.6x10^0$	--	--
15	15	6.9	598.3	--	--	--	30	0.5	--	--	--	--	15	0.15	--	--	--	--	$160x10^0$	$21x10^0$	$23x10^0$
16	8	6.5	688.8	--	--	--	4	--	--	--	--	--	--	--	--	--	--	--	$93x10^0$	$3.6x10^0$	--
17	9	7.0	598.6	--	--	--	45	0.4	0.05	9	0.4	--	6	0.5	--	--	--	--	$93x10^0$	--	--
18	8	6.4	593.6	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
19	8	6.8	682.9	--	--	--	4	--	--	--	--	--	--	--	--	--	--	--	$93x10^0$	--	--
20	8	6.8	682.9	--	--	--	20	0.5	--	2	--	--	1.5	--	--	--	--	--	$240\ x10^0$	$93\ x10^0$	--
21	8	6.8	692.8	9	--	--	26	0.1	--	5	--	--	4.5	--	--	--	--	--	$93\ x10^0$	--	$3.6x10^0$
22	9	6.7	681.4	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	$150\ x10^0$	--	$21\ x10^0$
23	8	6.8	596.7	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	$93\ x10^0$	--	$21\ x10^0$
24	8	7.0	677.2	--	--	--	3.5	--	--	--	--	--	--	--	--	--	--	--	$93\ x10^0$	$21\ x10^0$	--
25	8	6.7	512.7	6	--	--	7.5	1.4	--	9	0.1	0.01	5	0.2	--	--	--	--	$15\ x10^0$	$3.6\ x10^0$	--
26	10	6.9	666.3	--	--	--	130	1.8	--	16	1.4	--	78	3.4	--	--	--	--	$150\ x10^0$	$9.2\ x10^0$	$9.2\ x10^0$
27	9	6.7	583.3	--	--	--	9	0.1	--	3	--	--	10	--	--	--	--	--	$7.4\ x10^0$	$3.6\ x10^0$	--
28	3	7.1	483.6	--	--	--	13	1.3	0.01	4	--	--	2.5	--	--	--	--	--	$14\ x10^0$	--	$3.6\ x10^0$
29	5	6.8	567.3	--	--	--	78	0.1	--	--	24	--	58	2.7	--	--	--	--	$20\ x10^0$	$9.2\ x10^0$	--
30	5	6.8	385.9	--	--	--	60	0.8	--	--	7	--	90	3.5	--	--	--	--	$15\ x10^0$	--	$3.6\ x10^0$

AºD, Doric grade acidity; pH, potential of hydrogen; K, energy content; CFU/ml, colony forming units/milliliter; MPNg/ml, most likely number gram/milliliter; VB, bright green medium; EC, medium *Escherichia coli*. Note: Sample 9 was discarded because it was a raw EHM.

in 17.24% of samples. The presence of 82.76% of coliforms was detected in the presumptive test. In the confirmatory VB test, the presence of 54.16% of total coliforms was found and, in the EC test, we verified 33.33% of thermotolerant coliforms. However, in 12.5% of EHM samples tested positive for total and fecal coliforms.

Table 2. Quantification of coliforms in the presumptive, confirmatory VB and EC phase with ºD acidity

Sample	A ºD	MPN g/ml - Coliforms		
		Presumptive	Confirmatory VB	Confirmatory EC
4	8	$23x10^0$	--	--
5	8	$9.2x10^0$	--	--
6	10	$23x10^0$	--	--
7	5	$240x10^0$	$21x10^0$	--
10	8	$21x10^0$	--	$21x10^0$
11	3	$21x10^0$	$21x10^0$	--
12	5	$3.6x10^0$	$3.6\ x10^0$	--
13	8	$3.6x10^0$	$3.6\ x10^0$	--
14	9	$3.6x10^0$	--	--
15	15	$160x10^0$	$21x10^0$	$23x10^0$
16	8	$93x10^0$	$3.6x10^0$	--
17	9	$93x10^0$	--	--
19	8	$93x10^0$	--	--
20	8	$240x10^0$	$93x10^0$	--
21	8	$93x10^0$	--	$3.6\ x10^0$
22	9	$150x10^0$	--	$21\ x10^0$
23	8	$93x10^0$	--	$21\ x10^0$
24	8	$93x10^0$	$21\ x10^0$	--
25	8	$15x10^0$	$3.6\ x10^0$	--
26	10	$150x10^0$	$9.1\ x10^0$	$9.2\ x10^0$
27	9	$7.4x10^0$	$3.6\ x10^0$	--
28	3	$14x10^0$	--	$3.6\ x10^0$
29	5	$20x10^0$	$9.2\ x10^0$	--
30	5	$15x10^0$	$3.6\ x10^0$	$3.6\ x10^0$

AºD - Dornic degree acidity; MPNg/ml - most likely number gram/milliliter; VB - bright green medium; EC - *Escherichia coli* medium

The percentage distribution of the other microorganisms (mesophiles, psychrophiles, thermophiles, filamentous and yeast-like fungi and *Staphylococcus* spp) in the HM samples as a function of quantification and their serial dilutions are shown in Table 3.

Table 3. Percentage distribution of the presence of microorganisms in human milk samples

Microorganism/Group	Percentage distribution of the samples according to quantification and their serial dilutions		
	$\vdash 10^0$	$10^0 \vdash 10^{-1}$	$10^{-1} \vdash 10^{-2}$
Psychrophiles	17.24%	3.45%	0%
Thermophiles	27.59%	17.24%	3.45%
Mesophiles	55.17%	44.83%	10.34%
Filamentous and yeast-like fungi	41.38%	20.69%	0%
Staphylococcus aureus	0%	0%	0%

The mesophilic microorganism showed a moderate positive correlation with Dornic acidity (r=0.44; p=0.01) (Figure 1). Pearson's linear correlation test in the present sample did not show statistically significant correlations between the other groups of microorganisms and Dornic acidity (p> 0.05).

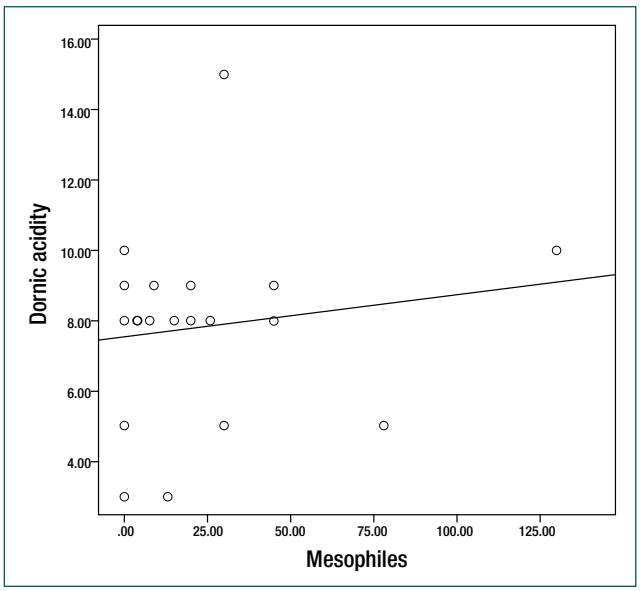

Figure 1. Linear correlation of °Dornic acidity and mesophiles (n=30)

Discussion

It was evidenced in this study that PEHM samples were composed by an oscillation of 3 to 15ºD, and 20.7% of them presented the acidity between 3 and 5ºD; 55.2% showed an acidity of 8ºD and 24.1% acidity between 9 and 15ºD. Under normal conditions, Dornic acidity in the PEHM varies from 2° to 7°D, and samples with acidity ≥ 8°D are considered unfit for consumption. Furthermore, it is known that the disposal of these samples occurs between the pasteurization phase and the microbiological analysis, that is, pasteurization aims to inactivate the microbial agents and Dornic acidity is a physical parameter and indicator of EHM hygienic quality.[12,16,20,32] For this study, the increase in Dornic acidity is in line with the findings of previous studies,[32,34] because the temperature rise after defrosting for the pasteurization process interacts as a titratable acidity modifier, a characteristic ob-

served in 13.79% of our samples, which identified 8ºD acidity and microbial absence after microbiological analysis. Our findings also corroborate with another study that, during EHM storage, lipid oxidation occurred, increasing acidity,[25] and 86.20% of samples showed unfeasibility due to the presence of microorganisms. Another study also demonstrated that the increase in Dornic acidity is caused by the degraded lactic acid of lactose through microbial action.[19]

In our study, we obtained an average energy value (K) of the analyzed samples of 587 Kcal/l, SD=77.4 Kcal/l. Energy content is a set of results from the individual characteristics of donors, from the lactation period, until losses during the storage process and from the milk sample itself (milk at the beginning and/or end of the feeding).[19] The pH mean obtained in the analyzed PEHM samples was 6.8 and this value corroborates the findings of another study that obtained a variation for PEHM of 6.86 (SD=0.24).[34] In our samples, we did not detect a decrease in pH in relation to high acidity and even with the microbial presence, probably due to the destabilization of casein making calcium less available, a phenomenon that is related to the inadequate conservation of HM after collection.[19] Another study highlights, in addition to the sanitary hygienic quality, that the titratable acidity is related to the storage in refrigeration at 4ºC in which lipolytic and proteolytic reactions occur, releasing fatty acids and free amino acids, resulting in a decrease in pH and an increase in acidity.[25]

In 82.76% (n=24) of our samples, the presence of coliforms in the presumptive test with the value of 3,600 and 240,000 MPN/ml was evidenced. In the confirmatory VB test, the presence of 54.16% of total coliforms was found; in the EC test, we verified 33.33% of thermotolerant coliforms and 12.5% showed a positive test for total and thermotolerant coliforms. This is a relevant factor considering that its presence is a classic indicator for the detection of coliforms and indicates that there has been a breach of good practices in handling HM, including inadequate collection techniques, poor hygiene of donors and utensils, and maintenance of the milk outside the cold chain.[16,35] In addition, the high initial microbial load compromises the efficiency of pasteurization. Contrary to our results, a study obtained the presence of coliforms in 10% (n=2) of the analyzed samples of PEHM,[34] which corroborates the results of another research[35] in which assessment of PEHM's hygienic sanitary conditions revealed the presence of 5.6% (n=8) total coliforms and 25.9% of thermotolerant coliforms that suggested fecal contamination.[24]

In our study, we identified the presence of psychrophilic microorganisms in 17.24% and thermophiles in 27.59% in the samples of PEHM. One study showed the presence of psychrophiles in 36.7% of their PEHM samples and 6.7% of thermophilic microorganisms,[36] suggesting secondary contamination, with inadequate refrigeration handling or storage.

Our findings revealed 55.17% of mesophiles and $4x10^1$ to $130x10^1$ CFU/ml. It should be noted that the allowable amount of aerobic mesophilic microorganisms is up to $1x10^2$ CFU/ml.[35] These findings are higher than the values found by another study[24] in which 48.2% of mesophiles were identified. In contrast, another study[37] evidenced the presence of 96% of this microorganism in its analyzes, corroborating the findings of three other studies[38–40], which found a population of 96% and 98.6% of mesophiles in their samples.[39] In addition, in our study, specifically the aerobic mesophilic group, after Pearson's linear correlation test, demonstrated a positive correlation with Dornic acidity (r=0.44; p=0.01).

The detection of mesophilic microorganisms in HM samples higher than the reference value shows contamination caused by the absence of good handling practices. Mesophilic bacteria act as an indicator of HM's health quality.[27,38] Additionally, the presence of these microorganisms is related to inadequate collection techniques, poor hygiene in relation to donors and utensils as well as the maintenance of milk outside the cold chain.[5,27,34,38,40]

We also identified 41.38% of filamentous and yeast-like fungi in our samples. Researchers found a prevalence of filamentous and yeast-like fungi in 69.4% of their samples.[41] In another study, the presence of filamentous and yeast-like fungi was re-

ported in 56% of their samples.[39] The incidence of filamentous and yeast-like fungi suggests transfer to the hands of donors and consequently to EHM, a fact of extreme relevance for the control of asepsis and proper handling during the collection of EHM in order to avoid contamination.[33]

It is noteworthy that, in our study, no microbial growth of *Staphylococcus* spp was found, similar to the other two studies with 20 and 14 samples of PEHM.[34,40] This microorganism is part of the group of mesophilic bacteria having an optimal growth at 37°C and in the range between 7 to 47.8°C, in addition to presenting a microbial growth at a pH of 4 and 9.8. Therefore, the presence of this microorganism in HM is indicative of an external contaminant, coming from manipulators, utensils and equipment.[21,36,40]

We recognize some limitations of this study, such as the small sample size that made it impossible to conduct subgroup analyzes and multivariate analyzes. Another limitation is that most of the previous studies in literature reflect on raw EHM and our study used EHM, which may have made some comparisons difficult. However, this study, unlike those previously published, used the group of microbiological microorganisms that indicate hygiene conditions in food through temperature variation, not choosing to classify it.

Conclusion

Based on the microbiological quality assessment of PEHM samples discarded and considered unfit for consumption in the referred HMB, specifically in relation to the microbiological indicators of hygiene conditions, it is suggested that the infeasibility of the samples may be associated with good food handling practices. Among them are inadequate collection techniques, inadequate preservation of EHM after collection, and secondary contamination, with inadequate handling or storage of refrigeration, which may have been caused by both donors and the HMB itself. It is hoped with our findings they can contribute to managers' and health professionals' reflection, highlighting the need for continuing education with HMB, in order to improve good practices in handling HM, especially in line with the sanitary hygiene quality in its collection, since it does not exercise absolute monitoring in external collection.

Collaborations

Oliveira C, Lopes-Júnior LC and Sousa CP contributed to the study design, data analysis and interpretation, writing of article, relevant critical review of intellectual content and approval of the final version to be published.

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