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Effect of bactofugation of raw milk on total bacterial and aerobic spore counts and on microbial diversity of sporeformers

Efeito da bactofugação do leite cru nas contagens bacteriana total, esporos aeróbios e na diversidade microbiana de formadores de esporos

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Highlights

Bactofugation can promote the reduction of 99.52% in TBC.
Significantly lower (95%) aerobic spores counts in bactofuged milk.
Bactofugation of milk can reduce the diversity of sporeformers.
Unusual dominant sporeformers, like *B. toyonensis* and *L. fusiformis.*

Abstract

This study describes the effect of bactofugation (10,000 × g in a continuous flow of 10,000 L/h) of three batches of raw milk on total bacterial count (TBC) and aerobic spore count, and it also shows the effect on the microbial diversity of spore-forming bacteria by analysing their genetic variability through molecular approaches. Given that milk must be preheated to approximately 55 °C before being bactofuged, for comparison, the three batches were evaluated at different stages as refrigerated raw, preheated, and bactofuged milk. For preheated milk, it was found that bactofugation caused a significant reduction ($p < 0.05$) of 99.52% (from 4.5×10^6 to 2.1×10^4 CFU/mL) in the mean TBC and of 95% (from 333 to 17 CFU/mL) in the aerobic mesophilic spore count. Due to the effect of bactofugation on preheated milk, a reduction of 82% was observed in both TBC and aerobic spore count. With respect to diversity, 107 isolates from raw milk, prior to bactofugation, and 16 isolates from bactofuged milk were recovered and grouped into 40 and 8 clusters, respectively. The predominant species detected in raw and preheated milk were *Bacillus toyonensis* (63% - 20 clusters) and *Lysinibacillus fusiformis* (15% - 8 clusters). *Proportionally, B. toyonensis* (69% - 6 clusters) and *L. fusiformis* (25% - 1 cluster) were predominant in bactofuged milk. *B. pumilus, L. varians, B. flexus, B. invictae,* and *B. megaterium*, bacteria with a known milk spoilage potential, were isolated from milk prior to bactofugation, and they reduced to undetectable levels in bactofuged milk. Bactofugation of milk, therefore, reduces the TBC and aerobic spore count, with a significant effect in reducing the microbial diversity of spore-forming bacteria, proportional to their incidence in raw milk. Therefore, bactofugation can be an alternative to increase the shelf life and technological potential of milk.

Key words: Bactofuga. Genetic variability. Shelf life. Spores.

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Resumo

Esse estudo descreve o efeito da bactofugação (10,000 × g em fluxo contínuo de 10,000 L/h) de três lotes de leite cru nas contagens bacterianas totais (CBT) e de esporos aeróbios, verificando também o efeito sobre a diversidade microbiana dos formadores de esporos por abordagem molecular de variabilidade genética. Como o leite a ser bactofugado deve ser pré-aquecido (≈55ºC), os três lotes foram avaliados enquanto cru refrigerado, pré-aquecido e bactofugado. Em associação com o pré-aquecimento, foi verificado que a bactofugação promoveu a redução significativa ($p < 0.05$) de 99,52% (4,5 x 10^6 para 2,1 x 10^4 UFC/mL) na contagem bacteriana total e 95% (333 para 17 UFC/mL) dos esporos aeróbios mesófilos. Considerando o efeito isolado da bactofugação sobre o leite já pré-aquecido, foi observada a redução de 82% tanto para CBT quanto para formadores de esporos. Em relação à diversidade, foram recuperados 107 isolados do leite anterior à bactofugação e 16 isolados do leite bactofugado, agrupados em 40 e 8 clusters, respectivamente. Foi observado predomínio das espécies *Bacillus toyonensis* (63% - 20 clusters) e *Lysinibacillus fusiformis* (15% - 8 clusters) no leite cru e pré-aquecido e, proporcionalmente, *B. toyonensis* (69% - 6 clusters) e L. fusiformis (25% - 1 *cluster*) no leite bactofugado. *Bacillus pumilus, Lysinibacillus varians, B. flexus, B. invictae,* e *B. megaterium*, isolados do leite antes da bactofugação e com potencial deteriorante conhecido, foram reduzidos a níveis indetectáveis no leite bactofugado. A bactofugação do leite, portanto, reduz a CBT e as contagens de esporos aeróbios, também com efeito significativo na redução da diversidade de formadores de esporos, proporcionalmente, conforme a sua incidência no leite cru, tendo potencial para ser utilizado como alternativa para aumento da vida útil e potencial tecnológico do leite.
Palavras-chave: Bactofuga. Esporos. VIDA útil. Variabilidade genética.

Introduction

The introduction of spore-forming microorganisms is difficult to control while obtaining milk. These microorganisms may potentially compromise the shelf life of pasteurised milk by late spore germination (Huck, Hammond, Murphy, Woodcock, & Boor, 2007) or even promote unwanted changes in cheese during production (Oliveira et al., 2016).

A bactofuge is a high-speed centrifuge specially designed to remove bacterial spores from milk at high temperatures (Farkye, 2004). This equipment centrifuges milk at approximately 9,000 × g for at least 1 s at 55 to 60 °C (Gésan-Guiziou, 2010) and removes the bactofuged concentrate containing the bacterial spores (Stack & Sillen, 1998). In some industries, bactofuged residues are thermally processed and re-incorporated into bactofuged milk to reduce potential volume losses.

Preliminary studies have demonstrated the efficiency of bactofugation in reducing gram-negative bacteria (Faccia, Mastromatteo, Conte, & Del Nobile, 2013) and bacterial spores in milk used for cheese production (Kosikowski & Fox, 1968; Torres-Anjel & Hedrick 1971). However, little is known about the effect of bactofugation of raw milk on total bacterial counts and on the microbial diversity of bacterial spores. Therefore, our objective was to verify the effect of bactofugation of raw milk on total bacterial count (TBC) and on aerobic spore count along with the interference it promotes in microbial diversity, using a microbiological and molecular approach.

Material and Methods

Three different batches of raw milk (A, B, and C) were evaluated immediately before processing in a cheese factory in São Paulo state, Brazil. Milk batches were collected from different dairy farms and stored in different bulk tanks. As the bactofugation process must be carried out after heating the milk (at approximately 55 °C), the batches were evaluated at three different stages, when milk was still raw (before preheating), after preheating (immediately before bactofugation), and after being bactofuged.

Refrigerated raw milk was collected from the bulk tanks. The remaining samples from the batch were collected at the entrance (preheated samples) and exit (bactofuged samples) of the bactofuge after the asepsis and installation of hermetically closed sanitary taps in the pipes of the unit. The bactofuge (Padroniza Indústria Brasileira de Pasteurizadores Ltda., Bauru, São Paulo) installed in the cheese factory had a centrifugal force of 10,000 × g with a continuous flow of 10,000 L/h. The concentrated bactofugate was discarded (less than 1% of total volume). The milk samples were sent to the Laboratory of Inspection of Animal Products of the National Institute of Science and Technology for the Milk Production Chain, headquartered at the State University of Londrina, Paraná State, Brazil. The interval between collection and microbiological analyses did not exceed 4 h.

For TBC or mesophilic aerobic spore count determination, serial dilutions (up to 10-^{4}) of the milk samples were performed in sterile peptone (0.001%) saline (0.85%) solution, and duplicates were surface-plated (0.1 mL) on plate count agar (Oxoid, Basingstoke, UK). Plates were incubated at 35 ± 1 °C for 48 h. The aerobic spore count in the milk was determined according to the Standard Methods for the Examination of Dairy Products. In accordance with Frank and Yousef (2004), 200 mL samples were subjected to 80 °C (± 0.5) for 12 min to remove the bacterial spores and stimulate spore germination. After the thermal treatment, the samples were serially diluted (up to 10^{-2}) in sterile peptone (0.001%) saline (0.85%) solution, followed by surface plating in duplicates (0.1 mL) in plate count agar (Oxoid) supplemented with 0.1% soluble starch (Synth, São Paulo, Brazil). Plates were inverted and incubated for 48 h at 32 ± 1 °C. The results of the counts were submitted to nonparametric statistical analyzes by Chi-Square test in SAS software v. 9.0 (SAS Institute, Inc., Cary, NC, USA).

All colonies of spore-forming bacteria from one of the duplicate plates were recovered in brain heart infusion broth (Acumedia, Baltimore, MD, USA), purified on plate count agar, and subjected to DNA extraction. All extracts were subjected to amplification of the internal transcribed spacer (ITS) region between 16S and 23S rRNA genes using the protocols, primers, and conditions previously reported (Ribeiro, Tamanini, Alfieri & Beloti, 2020).

The amplicons of the ITS region were subjected to restriction with 2 U of the enzyme HhaI (Invitrogen, CA, USA) using the reaction protocol described by the manufacturer (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Anza59_HhaI_PI.pdf). The amplified DNA and enzyme mixtures were incubated for 1 h at 37 °C in a thermocycler.

The amplification profiles of the ITS regions of each isolate, together with the HhaI restriction products, were used as genomic variables to construct a dendrogram of phylogenetic similarity using the Bionumerics software (v1.50, Applied Mathematics, Kortrijk,

Belgium). The similarity matrix Dice coefficient and the unweighted pair group mean algorithm were also used. To determine clusters, a minimum of 60% phylogenetic similarity was used.

A representative sample from each cluster was selected for partial amplification of the 16S rRNA gene using the primers and conditions described by Ribeiro et al. (2020). The polymerase chain reaction products were purified (PureLink Genomic DNA Purification Kit, Invitrogen) and quantified (Qubit dsDNA HS Assay Kit, Invitrogen) for DNA sequencing using the Sanger method (ABI 3500 Genetic Analyser, Applied Biosystems, CA, USA), which was completed in both directions.

The quality of the sequences was evaluated using the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and the consensus sequences were generated by CAP 3. These sequences were individually aligned using ClustalW and the representative sequences of each genus available in the Ribosomal Database Project (https://rdp.cme.msu.edu/). The genetic similarity was analysed via the neighbour-joining method and the Tamura-Nei model using 1,000 bootstrap replications in the MEGA X software (https://www.megasoftware.net/).

Results and Discussion

The TBC and aerobic spore counts for raw, preheated, and bactofuged milk, from batches A, B, and C, are shown in Table 1. Considering the mean counts of the three batches, it was possible to verify that bactofugation, for preheated milk, caused a significant reduction ($p < 0.05$) of 99.52% (from 4.5×10^6 to up to 2.1×10^4 CFU/mL) in TBC and of 95% (from 333 to up to 17 CFU/mL) from bacterial spores.

Table 1
Total bacteria and aerobic spores in milk batches at different stages of processing

Batch	Total Bacterial Count (CFU/mL)			Spore-forming bacteria (CFU/mL)		
	Raw	Preheated	Bactofuged	Raw	Preheated	Bactofuged
A	3.4×10^6	2.4×10^5	2.9×10^4	200	150	5
B	8.3×10^6	1×10^5	1.7×10^4	250	40	5
C	1.7×10^6	2×10^4	1×10^4	550	100	40
Mean*	4.5×10^{6a}	1.2×10^{5a}	2.1×10^{4b}	333^c	97^c	17^d

* Values followed by different superscript letters are significantly different according to $\chi 2$ tests.

Bactofugation alone, considering the counts of bactofuged milk in relation to preheated milk, caused a reduction of 82% in both TBC and aerobic spores. Torres-Anjel and Hedrick (1971) demonstrated that bactofugation of raw milk can reduce spore counts from 98% to > 99%, a result similar to the 95% observed in the present study. We recently described the efficiency of bactofugation in reducing 89.66% of preheating resistant psychrotrophs from raw milk (Ribeiro et al., 2019) and 88% of mesophilic thermoduric bacteria (Ribeiro et al., 2020).

Regarding the microbial diversity of spore-forming bacteria, 107 isolates from raw milk from all batches were recovered. These isolates were grouped into 40 clusters using the genetic variables of the amplification profile of the ITS region and their respective restriction with the *Hha*I enzyme (Figure 1). From the batches of bactofuged milk, 16 isolates were recovered and grouped into eight clusters (Figure 2).

Table 2 depicts the genetic similarity dendrograms as identified by sequencing the 16S rRNA gene of a representative isolate from each cluster. In all raw milk batches, eight different species were identified, with a predominance of *Bacillus toyonensis* (63% - 20 clusters) and *Lysinibacillus fusiformis* (15% - 8 clusters). A representative sequence of each identified species was deposited in GenBank with access numbers MT598179 to MT598186 (Table 2). Accordingly, in isolates from the bactofuged milk samples, *B. toyonensis* (69% - 6 clusters) and *L. fusiformis* (25% - 1 cluster) were also predominant.

As well as *L. fusiformis*, which we also describe as the highest incidence among bactofuge-resistant mesophilic thermodurics (Ribeiro et al., 2020), *B. toyonensis* is also not a commonly reported sporeformer from raw milk. This may be related to the composition of the microbiota of this sampling unit or, even, Huang, Flint, Yu, Ding, & Palmer (2021) study recently also described the isolation of *B. toyonensis* from dairy products with biofilm potential, in addition our potential to sporulate and produce toxins. The biofilms in the installations of bulk tanks, trucks and pipelines, from milking to dairy company, can be the origin of the predominance of this species in the milk samples of this study.

It was observed that the less frequent species in raw milk (Bacillus pumilus, Lysinibacillus varians, B. flexus, B. invictae, and B. megaterium) were reduced to undetectable levels in bactofuged milks and that the most frequent species in milk before bactofugation (B. toyonensis, L. fusiformis, and B. licheniformis) were the only ones identified in bactofuged milk samples. Among the mesophilic thermodurics, we have also reported that the species Macrococcus caseolyticus, Lysinibacillus varians, Carnobacterium divergens, Microbacterium hominis, Kocuria indica, Micrococcus yunnanensis, Gordonia paraffinivorans, Bacillus invictae, and Kocuria kristinae can be reduced to undetectable levels by raw milk bactofugation (Ribeiro et al., 2020).

In the state of Paraná, Brazil, we also describe the main sporeformers with spoilage potential, identifying the genus Bacillus, Paenibacillus e Brevibacillus and the species B. licheniformis as predominant (Ribeiro et al., 2018). B. licheniformis is aerobic spore bacteria frequently isolated from raw milk in several countries, such as Belgium (Scheldeman, Pil, Herman, De Vos, & Heyndrickx, 2005; Coorevits et al., 2008), Uruguay (Reginensi et al., 2011), China (Yuan et al., 2012) and the United States (Buehner, Anand, & Garcia, 2014), and the spores of these bacteria can be spread through air (Tortora, Funke, & Case, 2007). Due to its known proteolytic and/or lipolytic activity (Ribeiro et al., 2018) and thermoduric potential (Ribeiro et al., 2020), this species is especially important for dairy products. From the results of this study, we demonstrate that B. licheniformis can be reduced in quantity (10 to 1) and diversity (4 to 1 cluster) by milk bactofugation.

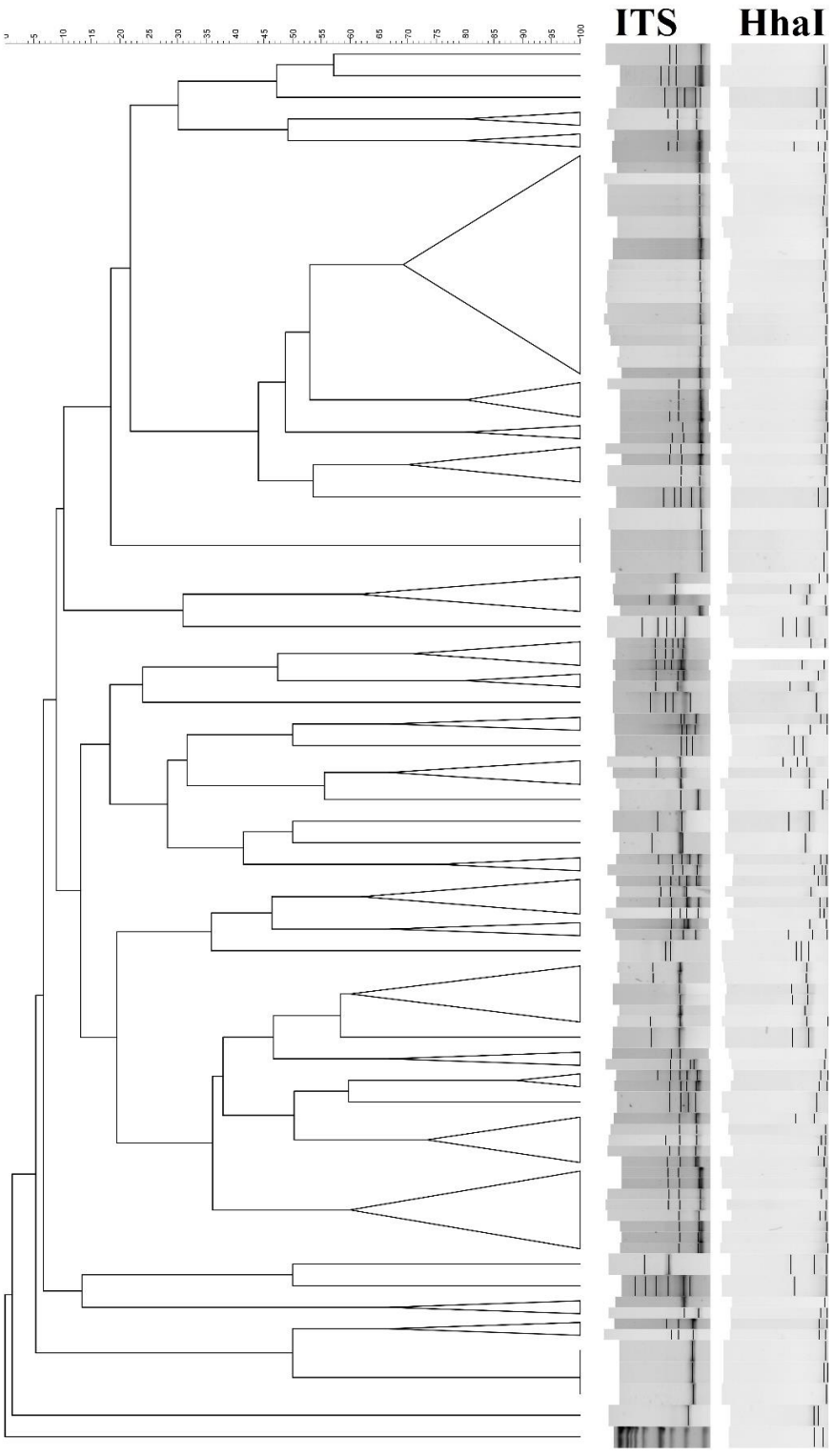

Figure 1. Dendrogram showing genetic similarities among spore-forming bacteria isolated from three batches of refrigerated raw milk prior to bactofugation, based on their ITS region amplification and *Hha*I restriction profiles. In total, 40 clusters with 60% genetic similarity are observed.

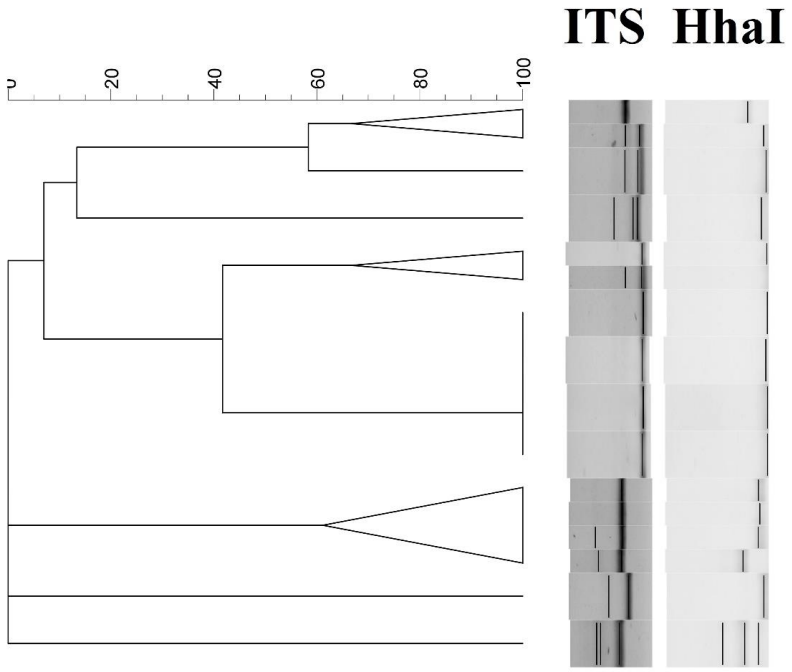

Figure 2. Dendrogram showing genetic similarities among spore-forming bacteria isolated from three batches of preheated and bactofuged milk, based on their ITS region amplification and *Hha*I restriction profiles. In total, eight clusters with 60% genetic similarity are observed.

Table 2
Identification, by clusters of genetic similarity, of spore-forming bacteria isolates from different milk batches before and after bactofugation

16S rRNA gene clustering identification	Accession number of a representative strain	Raw milk			Bactofuged milk		
		Cluster	n	Batch (n)	Cluster	n	Batch (n)
Bacillus toyonensis	MT598179	I	1	C	I	2	C
		II	1	C	II	1	C
		V	2	A (1) B (1)	III	1	A
		VI	21	A (4) B (1) C (16)	IV	2	C
		VII	4	C (4)	V	4	A (1) C (3)
		VIII	2	C (2)	VIII	1	B
		XI	3	A (1) B (1) C (1)			

continuation...

		XIII	1	B (1)			
		XXVI	1	B (1)			
		XXI	1	A (1)			
		XXII	1	C (1)			
		XXIV	4	A (2) B (1) C (1)			
		XXVIII	1	A (1)			
		XXIX	2	B (1) C (1)			
		XXXII	5	B (2) C (3)			
		XXXIII	8	C (8)			
		XXXV	1	C (1)			
		XXXVI	2	A (1) B (2)			
		XXXVIII	3	B (3)			
		XL	1	B (1)			
Bacillus licheniformis	MT598180	III	1	A (1)	VII	1	B
		IV	2	B (1) C (1)			
		X	1	A (2)			
		XXVII	6	A (6)			
Lysinibacillus fusiformis	MT598181	IX	4	C (4)	VI	4	B
		XIV	3	A (3)			
		XVII	2	A (2)			
		XVIII	1	A (1)			
		XXIII	2	A (1) C (2)			
		XXV	2	A (2)			
		XXXIV	1	C (1)			
		XXXIX	1	B (1)			
Bacillus pumilus	MT598182	XV	2	A (2)			
		XX	1	B (1)			

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Lysinibacillus varians	MT598183	XVI	1	A (1)
		XXXI	1	C (1)
Bacillus flexus	MT598184	XIX	3	A (2) B (1)
Bacillus invictae	MT598185	XXX	2	A (1) B (1)
Bacillus megaterium	MT598186	XXXVII	2	A (1) C (1)

Conclusion

Milk bactofugation can reduce the microbial diversity of spore-forming bacteria proportionally to their incidence in raw milk. Thus, this method has the potential to be used for the quantitative reduction of spore-forming bacteria. This method can also promote the reduction of TBC and microbial diversity of microorganisms with predicted or known spoilage potential, leading to an increase in the shelf life and improving the technological potential of bactofuged milk.

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