Effect of milk bactofugation on the counts and diversity of thermophilic bacteria

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ABSTRACT

The objective of this work was to determine the effect of milk bactofugation on the counts and microbial diversity of mesophilic (MT), psychrotrophic (PT), and thermophilic (TT) thermophilic bacteria and its potential as a technological method to remove spoilage microorganisms resistant to pasteurization. Different batches of raw milk from 69 dairy farms divided into sets in 3 bulk tanks (A, B, C) were evaluated at different times during the technological process. As the raw milk was preheated ($\sim 55^{\circ}\text{C}$) immediately before bactofugation ($10,000 \times g$), the effect of bactofugation was estimated by comparing the counts in raw, preheated, and bactofuged milk. This centrifugation was sufficient to reduce the isolation of 88% of the MT in preheated milk. For PT, it was possible to verify a reduction of 72.5% in batch C. The TT were not recovered at higher detection limits ($<5 \text{ cfu/mL}$). For diversity, 310 isolates were identified using a molecular approach; 15 species of contaminating thermophilic bacteria were identified from raw and preheated milk, and only 6 species were recovered in bactofuged milk. Only MT were recovered from the bactofuged milk, mainly the species *Lysinibacillus fusiformis* (61.7%) and *Bacillus licheniformis* (12.3%). Both species are known to be endospore-forming psychrotrophs and have proteolytic or lipolytic activity. The bactofugation of raw milk reduced the number of isolates of *B. licheniformis*, *Bacillus toyonensis*, *Micrococcus aloeverae*, and *Aestuariimicrobium kwangyangense* by 33, 43, 86, and 92%, respectively, and reduced the isolates of *Macroccoccus caseolyticus*, *Lysinibacillus varians*, *Carnobacterium divergens*, *Microbacterium hominis*, *Kocuria indica*, *Micrococcus yunnanensis*, *Gordonia paraffinivorans*, *Bacillus invictae*, and *Kocuria kristinae* to undetectable levels. The results of this study indicate that bactofugation can be

applied by the dairy industry to reduce pasteurization-resistant microorganisms in combination with prophylactic measures to prevent the contamination of raw milk by spores and vegetative forms of bacteria.

Key words: bactofugation, endospore-forming bacteria, high centrifugation, *Lysinibacillus fusiformis*

INTRODUCTION

Thermophilic bacteria are microorganisms capable of resisting pasteurization (Huck et al., 2007). Due to the proteolytic or lipolytic activity of these bacteria, they are often responsible for reducing the shelf life of pasteurized milk (Durak et al., 2006; Ribeiro Júnior et al., 2018a). Reducing the microbiological contamination of raw milk is essential to extend the shelf life of pasteurized milk (Murphy et al., 2016; Ribeiro Júnior et al., 2019a).

However, endospore-forming microorganisms, for example, which are difficult to control during milk production, can compromise the shelf life of pasteurized milk by spoilage activity of vegetative forms after spore germination (Huck et al., 2007) or promote undesired changes to cheese production (Oliveira et al., 2016). The vegetative forms of endospore-forming bacteria can be thermophilic (Ribeiro Júnior et al., 2018a) as well as other bacteria that do not form spores (Delgado et al., 2013; Ribeiro Júnior et al., 2018a).

Thus, the use of methods to reduce the number of bacterial endospore-forming agents in raw milk can be used to extend the shelf life of pasteurized milk and reduce the technological problems of dairy products produced with pasteurized milk. The study by Khanal et al. (2014), for example, reports that the ultrasonication of milk can eliminate the vegetative forms of the thermophilic bacteria of the genus *Bacillus*.

One of these methods, bactofugation, is the use of a high-speed centrifuge specifically designed to remove bacterial spores from milk at high temperatures (Farkye, 2004). The bactofuge centrifuges the milk at approximately $9,000 \times g$ for less than 1 s at 55 to 60°C (Gésan-Guizieu, 2010), thereby removing the concen-

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trated bacterial pellet that contains spores (Stack and Sillen, 1998).

Many studies have evaluated the use of this technology only to reduce bacterial spores and have reported its efficiency in reducing gram-negative bacteria, mainly for cheese production (Kosikowski and Fox, 1968; Torres-Anjel and Hedrick 1971; Faccia et al., 2013), which has limited its use on a large scale due to its applicability in only a few situations. Little is known about if this method is able to remove vegetative forms of spoilage microorganisms of raw milk that remain after pasteurization. Our objective, therefore, was to verify the effect of bactofugation of raw milk on the counts and microbial diversity of mesophilic (**MT**), psychrotrophic (**PT**), and thermophilic (**TT**) thermophilic bacteria.

MATERIALS AND METHODS

Three different batches of raw milk (A, B, C) were evaluated immediately before processing by a dairy company in the state of São Paulo, Brazil. Each batch was stored in an individual bulk tank. Batch A was composed of approximately 15,000 L (from 12 farms), B had 20,000 L (26 farms), and C had 11,000 L (31 small dairy farms). Thus, milk from 69 different dairy farms was evaluated in 3 sets. As the bactofugation process is carried out after milk heating (~55°C), the batches were evaluated in 3 stages during processing: raw, preheated (the condition immediately before bactofugation), and bactofuged milk.

Refrigerated raw milk was collected from the bulk tanks. The remaining samples from the batch were collected after the installation and asepsis of sanitary taps in the pipe in the entrance (preheated) and exit of the bactofuge that were hermetically closed. Aseptic sampling (~500 mL) of each batch was performed continuously during processing to ensure it was representative of the entire batch. The bactofuge installed in the cheese factory (Padroniza Indústria Brasileira de Pasteurizadores Ltda., Bauru, São Paulo, Brazil) had a force of 10,000 × *g* with a continuous flow of 10,000 L/h. The concentrated bactofugate was discarded (less than

1%). The milk samples were sent under refrigeration 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 in Paraná state (Brazil). The interval between collection and the microbiological analyses did not exceed 4 h.

Thermophilic bacteria were enumerated after heating milk (5 mL) to a temperature that simulates pasteurization (62.8 ± 0.5°C for 30 min) followed by immediate refrigeration at 10°C, keeping with the findings obtained by Frank and Yousef (2004). After treatment, serial dilutions of the samples were performed up to 10⁻³ 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 for MT, 7 ± 1°C for 10 d for PT, and 55 ± 1°C for 48 h for TT. The results of the counts were submitted to nonparametric statistical analyses by χ^2 test in SAS software (v. 9.0; SAS Institute Inc., Cary, NC).

All colonies from one of the duplicate plates were recovered in brain heart infusion broth (Acumedia, Baltimore, MD), purified on plate count agar, and subjected to DNA extraction according to the protocol of Ribeiro Júnior et al. (2016). All extracts were subjected to amplification of the internal transcribed spacer (**ITS**) region of 16–23S rRNA using the primers and conditions as described in Table 1. We performed PCR with approximately 50 ng of DNA template, 100 nM each dNTP, 5 µL of 10 × buffer, 75 mmol/L MgCl₂, 20 pmol/L of each primer, and 2.5 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA).

The amplicons of the ITS region were subjected to restriction with 2 U of the enzyme *HhaI* (Invitrogen) using the reaction protocol described by the manufacturer. 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 product, were used as genomic variables to construct a dendrogram of phylogenetic similarity (Ranjard et al., 2001) using Bionumerics version 1.50 software (Applied Mathemat-

Table 1. Primers and PCR cycling conditions

Gene	Primers (5'-3')	Size (pb)	PCR cycling conditions	Reference
<i>ITS</i>	CCGGGTTTCCCCATTCGG		94°C 5 min	Normand et al. (1996)
	AAGGAGGTGATCCAGCCGCA		40× (94°C 30 s, 60°C 30 s, 72°C 1 min) 72°C 5 min	
16S rRNA	GAGTTTGATCMTGGCTCAG GGYTACCTTGTTACGACTT	1,465	94°C 5 min 35× (94°C 1 min, 58°C 1 min, 72°C 1 min) 72°C 10 min	Edwards et al. (1989) Osborne et al. (2005)

The quality of the sequences was evaluated by the software BioEdit version 7.2.5 (Hall, 1999) and the consensus sequences were generated by CAP 3 (Huang and Madan, 1999). These sequences were individually aligned using Clustal W and the representative sequences of each genus available in the Ribosomal Database Project (<https://rdp.cme.msu.edu/>), and genetic similarity was analyzed via the neighbor-joining method and the Tamura-Nei model using 1,000 bootstrap replications in MEGA X software (Kumar et al., 2018).

If the microbiota of this sample unit had higher counts of PT and TT, it would be possible to verify the potential of bactofugation with more robust results.

Batches	Effect of bacto-fugation (%)														
	Raw			Preheated			Bacto-fugated			Raw milk			Preheated milk		
	MT	PT	TT	MT	PT	TT	MT	PT	TT	MT	PT	TT	MT	PT	TT
A	5.9×10^6	6.1×10^2	5	2.1×10^6	15	<5	2.4×10^5	<5	<5	96	98	<5	87	33	—
B	1.3×10^5	4.1×10^2	<5	6.5×10^4	<5	<5	2.5×10^4	<5	<5	80	93	<5	38	—	—
C	1.6×10^4	3.5×10^2	<5	3.5×10^3	40	<5	9.9×10^2	11	<5	94	97	<5	72	73	—
lean ¹	2.0×10^{6a}	4.5×10^2	5	7.2×10^{5a}	20	5	8.8×10^{4b}	7	5	96	95	5	88	65	—

values with different superscripted letters differed in the χ^2 test.

However, considering the results of the batches alone, in batch C, which had counts of PT both after preheating (40 cfu/mL) and after bactofugation (11 cfu/mL), a reduction of 72.5% was observed.

In a previous report, we already demonstrated that bactofugation has a significant effect on counts and on the diversity of psychrotrophs (Ribeiro Júnior et al., 2019b), reducing this microbiota by 89.66%. Psychrotrophs and thermodurics are the agents mainly responsible for the spoilage of raw and pasteurized milk, respectively (Mane and Gandhi, 2011; Ribeiro Júnior et al., 2018a). Avoiding contamination of raw milk by these microorganisms is essential to increase the technological potential of processed milk (Murphy et al., 2016). The use of bactofugation in this context can help to significantly reduce the contamination of milk by these microorganisms with spoilage potential, preserving the quality and integrity of the milk constituents.

Faccia et al. (2013) verified a reduction of *Enterobacteriaceae* and *Pseudomonas* in Mozzarella cheeses produced with bactofugated milk, and Kosikowski and Fox (1968) demonstrated the reduction of coliforms by

bactofugation in milk intended for cheese production. This can be explained by the low thermal resistance of gram-negative microorganisms to the preheating process. Torres-Anjel and Hedrick (1971) demonstrated that the bactofugation of milk can reduce spore counts 98 to >99%, a result similar to the 96% reduction in MT verified by the present study.

From the plates used in the counts, 310 thermoduric isolates were recovered. We isolated 229 MT, PT, and TT from the raw and preheated milk samples according to the distribution shown in Table 3. All of the thermoduric microorganisms isolated from the bactofuged milk (81) were mesophilic, and the distribution of these isolates by batch is shown in Table 4.

Although batch C had colonies of PT on the plates after bactofugation, which could be used to identify this microbiota, no isolates were recovered in the subsequent steps. It was also not possible to recover PT isolates (batches A and B) or TT isolates from bactofuged milk, which supports the results of counts <5 cfu/mL observed in Table 2. All TT were isolated from raw milk in batch A.

Table 3. Identification by cluster of genetic similarity of mesophilic (MT), psychrotrophic (PT), and thermophilic (TT) thermoduric isolates from batches of raw and subsequently preheated milk before bactofugation

16S rRNA clustering identification	Cluster	MT	PT	TT	Total n (%)
<i>Lysinibacillus fusiformis</i>	I	55	15		80 (34.9)
	VIII		1		
	XX			1	
	XXIV		7		
	XXV	1			22 (9.6)
<i>Micrococcus caseolyticus</i>	XIX	21	1		
<i>Micrococcus aloeverae</i>	III	15	7		
<i>Bacillus licheniformis</i>	V		1	2	
	XII	3			15 (6.6)
	XXII	7	1		
	XXIII		1		
<i>Lysinibacillus varians</i>	IX	6	6		
	X	1			15 (6.6)
	XV	1			
	XVI	1			
<i>Bacillus toyonensis</i>	II		2		
	XXVIII	4	1		14 (6.2)
	XXIX	3			
	XXX	1			
	XXXI		1		
	XXXII		1		12 (5.2)
<i>Aestuariaimicrobium kwangyangense</i>	XXXIII		1		
	XIV	8	4		
<i>Carnobacterium divergens</i>	VII	11	1		
<i>Microbacterium hominis</i>	XI	3	5		9 (3.9)
	XXVII	1			
<i>Microbacterium lacticum</i>	IV	7	1		
<i>Kocuria indica</i>	XIII	3	3		
<i>Micrococcus yunnanensis</i>	XVIII	6			6 (2.6)
<i>Bacillus invictae</i>	XXVI	2			
	VI			1	
<i>Gordonia paraffinivorans</i>	XXI		3		
<i>Kocuria kristinae</i>	XVII	1	1		2 (0.9)
Total (%)		161 (70.3)	64 (28)	4 (1.7)	229 (100)

Regarding microbial diversity, using the genetic variables of the amplification profile of the ITS region and its restriction with the *HhaI* enzyme, 33 (I–XXXIII) clusters of genetic similarity were observed for the 229 isolates of MT, PT, and TT from raw milk and preheated milk (Figure 1), and 12 (I–XII) clusters for the MT isolates from the samples of bactofuged milk (81; Figure 2). The identification of isolates by cluster is shown in Tables 3 and 4. Fifteen species of thermophilic contaminants from raw and preheated milk were identified, and only 6 species were recovered in the bactofuged milk.

It was found that *Lysinibacillus fusiformis* (80), *Macroccoccus caseolyticus* (22), and *Micrococcus aloeverae* (22) corresponded to 54% of the 229 thermophilic isolates from raw and preheated milk. There was also a predominance of endospore-forming bacteria (127/229 or 55%) among the isolates, corresponding to the species *L. fusiformis*, *Bacillus licheniformis*, *Lysinibacillus varians*, *Bacillus toyonensis* and *Bacillus invictae*.

According to Table 3, the isolates of *L. fusiformis*, which represented 35% of the thermophilic bacteria in raw and preheated milk, presented 5 distinct genetic types (clusters I, VIII, XX, XXIV, and XXV), which may have been determined by different sources of contamination for all evaluated batches. This species was isolated as a MT and PT from batches A, B, and C, and as a TT only in the raw milk sample from batch A. Additionally, in relation to the TT recovered from raw milk from batch A, the species *B. licheniformis* and *B. invictae* were also identified.

For the PT isolates of raw and preheated milk (n = 64), *L. fusiformis* (36%), *M. aloeverae* (11%) and *L. varians* (9%) predominated in addition to other species (Table 3). The *Gordonia paraffinivorans* species was notable as it represented 5% of the PT, was isolated exclusively as a psychrophilic microorganism, was an isolate obtained from each batch of milk (A,

B, and C), belonged to the same cluster (XXI), and demonstrated an origin common to the 3 batches (e.g., biofilms in a bulk transport vehicle or the water used in the cheese factory). The ability to form biofilms by microorganisms of this genus has been reported previously (Arenskötter, Bröker and Steinbüchel, 2004), and the species was described by Xue et al. (2003) as capable of degrading hydrocarbons. No previous reports were found regarding the isolation of this species in milk.

Table 4 demonstrates that among the microorganisms remaining for bactofugation, *L. fusiformis* and *B. licheniformis* species predominated (74%). Both species are known to be endospore-forming psychrophils and have proteolytic or lipolytic activity (De Jonghe et al., 2010; Miller et al., 2015; Pinto et al., 2018; Ribeiro Júnior et al., 2018b).

The bactofugation process was sufficient to reduce (37.5%) the isolation of *L. fusiformis* with mesophilic behavior from 80 (raw and preheated milk) to 50 (bactofuged milk), and to remove all microbes that had psychrophilic and thermophilic metabolism. We also reported that this species has the highest incidence among psychrophilic residues of bactofugation (Ribeiro Júnior et al., 2019b). Apparently, this greater recovery in bactofuged milk is proportionally linked to the higher incidence of this species in raw milk.

Similarly, bactofugation reduced the counts of *B. licheniformis*, *B. toyonensis*, *M. aloeverae*, and *Aestuariimicrobium kwangyangense* by 33%, 43%, 86%, and 92%, respectively, in addition to reducing isolates from *Macroccoccus caseolyticus*, *L. varians*, *Carnobacterium divergens*, *Microbacterium hominis*, *Kocuria indica*, *Micrococcus yunnanensis*, *G. paraffinivorans*, *B. invictae*, and *Kocuria kristinae* to undetectable levels. These measurements are probably affected by the resistance of each isolate to temperature and other intrinsic characteristics of the microorganisms.

Table 4. Identification by cluster of genetic similarity of mesophilic thermophilic residues of bactofugation from 3 batches of preheated milk

16S rRNA clustering identification	Cluster	n	Total (%)
<i>Lysinibacillus fusiformis</i>	X	48	50 (61.7)
	XI	1	
	VI	1	
<i>Bacillus licheniformis</i>	VIII	7	10 (12.3)
	IX	3	
<i>Microbacterium lacticum</i>	V	9	9 (11.1)
<i>Bacillus toyonensis</i>	III	4	8 (9.9)
	IV	1	
	VII	3	
<i>Micrococcus aloeverae</i>	I	2	3 (3.7)
	II	1	
<i>Aestuariimicrobium kwangyangense</i>	XII	1	1 (1.2)
Total			81 (100)

Figure 1. Dendrogram of genetic similarity of thermophilic bacteria isolated from 3 batches of raw and preheated milk before bactofugation, as constructed using the variable internal transcribed spacer (ITS) region amplification profile and the restriction of DNA from respective bacteria samples with the *HhaI* enzyme. Thirty-three clusters with 60% genetic similarity were observed; some are represented with numerals (I to XXXIII).

Figure 2. Dendrogram of genetic similarity of thermophilic bacteria isolated from 3 batches of raw and preheated milk after the bactofugation process, constructed using the internal transcribed spacer (ITS) region amplification profile variables and the restriction of DNA from respective bacteria samples with the *HhaI* enzyme. Twelve clusters can be observed with 60% genetic similarity, identified here from I to XII.

We emphasize that in this study we followed the *Standard Methods for the Examination of Dairy Products* (Frank and Yousef, 2004) for the thermophilic enumeration, which simulates vat pasteurization in analytical and laboratory conditions. Under industrial processing conditions using HTST pasteurization, the composition of the thermophilic microbiota could be changed.

CONCLUSIONS

The bactofugation of raw milk, preceded by preheating to 55°C, significantly reduced thermophilic bacteria counts. The MT remaining in the bactofuged milk was similarly reduced in diversity and quantity compared with those observed in the milk before bactofugation. Endospore-forming microorganisms with spoilage potential predominate among the thermophilic residues of bactofugation. Thus, this process can be used to reduce the microorganisms in raw milk resistant to pasteurization, preserving its quality, in association with other techniques that help control the microbiological contamination of raw milk by endospore-forming bacteria.

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