Short communication: Effect of bactofugation of raw milk on counts and microbial diversity of psychrotrophs

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ABSTRACT

Bactofugation is a centrifugal process for removing spores of microorganisms from milk, especially when it is destined for cheese making. Other microorganisms may be removed in bactofugation. This study aimed to verify the effect of milk bactofugation on the counts and microbial diversity of psychrotrophs. The raw milk was preheated ($\approx 55^{\circ}\text{C}$) before being bactofuged, and samples were collected from 3 batches of milk: refrigerated raw, preheated, and bactofuged, representing the immediate conditions before and after bactofugation. The mean psychrotrophic counts of the 3 batches were $3.08 (\pm 1.69) \times 10^6$, $193 (\pm 232)$, and $20 (\pm 26)$ cfu/mL, respectively. Preheating was sufficient to eliminate 99.99% of the raw milk psychrotrophs, but bactofugation further reduced 89.66% of psychrotrophs from preheated milk. *Lysinibacillus fusiformis* was the most frequently isolated species (45.7%) among the psychrotrophs of raw milk and, proportionally, were more frequent in preheated (37.5%) and bactofuged (60%) milk. *Bacillus invictae* (20%), *Enterococcus faecalis* (10%), and *Kurthia gibsonii* (10%) were also isolated from bactofuged milk. Albeit in small numbers, psychrotrophic, thermotolerant, and spore-forming bacteria with known proteolytic and lipolytic activity remained in the milk after bactofugation, which apparently had no effect on a specific population of microorganisms but proportionally reduced the entire psychrotrophic microbiota of raw milk.

Key words: bactofugation, bacterial count, efficiency, spoilage microorganisms

Short Communication

A bactofuge is a piece of equipment used by the dairy industry to reduce the microbial load of milk, especially when milk is of poor quality and intended for cheese production (Gésan-Guizieu, 2010). A bactofuge is a high-speed centrifuge specially 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), removing the concentrated bactofugate that contains spores (Stack and Sillen, 1998). In addition to sporulated microorganisms, counts of other microorganisms may also be reduced by bactofugation.

Milk psychrotrophs are potentially the major microorganisms responsible for milk deterioration (Sørhaug and Stepaniak, 1997). Lipases and proteases produced by most psychrotrophs significantly reduce the quality and shelf life of pasteurized and UHT fluid milks and dairy products (Dogan and Boor, 2003). Thus, the aim of the present study was to verify the effect of bactofugation of raw milk on the counts and the microbial diversity of psychrotrophs.

For this purpose, 3 different batches of milk were collected at different processing stages: raw, preheated (between 45 and 55°C), and bactofuged, totaling 9 milk samples. 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. The bactofuge installed in the cheese factory (Padroniza Indústria Brasileira de Pasteurizadores Ltda., Bauru, São Paulo, Brazil) had a force of $10,000 \times g$ with a continuous flow of 10,000 L/h. The concentrated bactofugate was discarded (less than 1%). The collections were carried out in September 2017 in a dairy company in the interior of São Paulo State, Brazil. 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,

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headquartered at the State University of Londrina, in Paraná State (Brazil). The interval between collection and the microbiological analyses did not exceed 4 h.

Serial decimal dilutions of milk samples were performed in saline solution (0.9%), which were seeded in duplicate on the surface of plate count agar (Oxoid, Basingstoke, UK). Plates were incubated at 7°C for 10 d. 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).

After the psychrotrophic bacterial counts, all colonies of 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 described by Normand et al. (1996) (F: CCGGGTTTCCCCATTCGG) and Edwards et al. (1989) (R: AAGGAGGTGATCCAGCCGCA). The PCR cycling conditions were 94°C for 5 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final step at 72°C for 5 min. The PCR was performed with approximately 50 ng of DNA template, 100 nM each dNTP, 5 μ L of 10 $\times$ buffer, 75 mmol/L MgCl₂, 20 pmol/L of each primer, and 2.5 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Amplification was performed in a thermocycler (Aeris Thermal Cycler, Esco Micro Pte. Ltd., Singapore), and the PCR-amplified DNA samples were loaded onto a 1% agarose gel (Invitrogen) and subjected to electrophoresis for 1 h at a constant voltage of 90 V. The gels were stained with 0.2 mg/mL ethidium bromide solution for 20 min before visualization. Images were saved after UV transillumination.

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 (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. Restriction products were subjected to agarose gel electrophoresis (1.5%) for 1 h at a constant voltage of 70 V. The gels were stained with 0.2 mg/mL ethidium bromide and documented.

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 v. 1.50 software (Applied Mathematics, Kortrijk, Belgium). The similarity matrix Dice coefficient (Dice, 1945) and the unweighted pair group mean algorithm (Sneath and Sokal, 1973) were 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 Osborne et al. (2005). The products of this PCR were purified (Pure-Link Genomic DNA Purification Kit, Invitrogen) and quantified (Qubit dsDNA HS Assay Kit, Invitrogen) for DNA sequencing using the Sanger method (ABI 3500 Genetic Analyzer, Applied Biosystems, Foster City, CA), which was completed in both directions.

The quality of the sequences was evaluated by the software BioEdit v. 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, and genetic similarity was analyzed via the neighbor-joining method and the Tamura-Nei model using 1,000 bootstrap replications in the MEGA v. 7.0 software (Kumar et al., 2016).

The mean ($\pm$ standard deviation) psychrotrophic counts of the 3 batches of raw, preheated, and bactofuged milk were $3.08 (\pm 1.69) \times 10^6$, $193 (\pm 232)$, and $20 (\pm 26)$ cfu/mL, respectively.

Preheated milk was the immediate condition before bactofugation and we observed a reduction of 89.66% in the psychrotrophic count between bactofuged and preheated milk ($P < 0.001$). Kosikowski and Fox (1968) showed that bactofugation of milk decreased bacterial numbers by 95.3%. They were the first to demonstrate the effect of bactofugation in the control of the microbiological quality of milk. The preheating for bactofugation ($\approx 55^\circ\text{C}$) alone was able to reduce the psychrotroph counts in raw milk by 99.99%. This is probably related to the predominance of thermolabile microorganisms.

In our analysis of microbial diversity, 64 psychrotrophic isolates were recovered: 46 from the 3 raw milk samples, 8 from the preheated samples, and 10 from the bactofuged milk samples. The dendrograms of genetic similarity between the isolates are shown in Figures 1 and 2; it can be seen that the isolates of the raw, preheated, and bactofuged milk were grouped into 14, 5, and 4 clusters, respectively, considering 60% genetic similarity.

Considering the identification by genetic similarity with the type species of the genus, microbial diversity is presented in Table 1. *Lysinibacillus fusiformis* was the most isolated species of psychrotroph in raw milk, accounting for almost half of the isolates. *Lysinibacillus fusiformis* is a sporulated microorganism isolated from milk (Coorevits et al., 2008), and it is expected to exhibit thermal resistance to preheating. Proportionally, it was also the most frequent species remaining after the

Figure 1. A genetic similarity dendrogram of psychrotrophic bacteria isolated from Brazilian raw milk, using the variables of the amplification profile of the internal transcribed spacer (ITS) region 16–23S and its restriction profile with the enzyme *HhaI*. The vertical line represents the minimum percentage of similarity (60%) used to determine the 14 clusters.

bactofugation process (60%; Table 1). Because isolates of this species have proteolytic and lipolytic potential (De Jonghe et al., 2010), any failure in storage conditions will compromise the quality of the bactofuged

milk and associated cheeses, even under refrigerated conditions.

Pseudomonas spp. are considered the main psychrotrophs of raw milk in several countries (Sørhaug and

Figure 2. A genetic similarity dendrogram of psychrotrophic bacteria isolated from (A) preheated and (B) bacto-fuged milk, using the variables of the amplification profile of the internal transcribed spacer (ITS) region 16–23S and its restriction profile with the enzyme *HhaI*. The vertical line represents the minimum percentage of similarity (60%) used to determine the clusters.

Table 1. Genetic diversity and proportionality [number (%)] of psychrotrophs isolated from 3 Brazilian milk batches at different stages of the process (raw, preheated, and bacto-fuged)

Species identification	Raw	Preheated	Bacto-fuged
<i>Lysinibacillus fusiformis</i>	21 (45.7)	3 (37.5)	6 (60)
<i>Bacillus toyonensis</i>	4 (8.7)	—	—
<i>Rhodococcus hoagii</i>	4 (8.7)	—	—
<i>Carnobacterium divergens</i>	3 (6.5)	—	—
<i>Microbacterium lacticum</i>	3 (6.5)	1 (12.5)	—
<i>Bacillus licheniformis</i>	2 (4.3)	2 (25)	—
<i>Buttiauxella brennerae</i>	2 (4.3)	—	—
<i>Pseudomonas</i> spp.	2 (4.3)	—	—
<i>Aestuariimicrobium kwangyangense</i>	1 (2.2)	—	—
<i>Buttiauxella gaviniae</i>	1 (2.2)	1 (12.5)	—
<i>Kocuria indica</i>	1 (2.2)	—	—
<i>Kurthia gibsonii</i>	1 (2.2)	1 (12.5)	1 (10)
<i>Micrococcus aloeverae</i>	1 (2.2)	—	—
<i>Enterococcus faecalis</i>	—	—	1 (10)
<i>Bacillus invictae</i>	—	—	2 (20)

Stepaniak, 1997; Marchand et al., 2009; Caldera et al., 2016; Vithanage et al., 2016; Xin et al., 2017). In Brazilian raw milk, we showed that *Pseudomonas* do not predominate (Ribeiro Júnior et al., 2018a). In the microbiota evaluated in the present work, *Pseudomonas* represented only 4.3% of raw milk psychrotrophs (Table 1), and the preheating process was sufficient to eliminate these microorganisms, as was also reported by Dogan and Boor (2003). Their proteases and lipases, however, are thermostable (Bagliniere et al., 2013) and may remain in bacto-fuged milk.

We previously described *Kurthia gibsonii* as a proteolytic and lipolytic psychrotrophic of Brazilian refrigerated raw milk (Ribeiro Júnior et al., 2018a). *Bacillus invictae* and *Enterococcus faecalis*, which were identified as psychrotrophic remnants of bacto-fugation in the present study, are thermotolerant microorganisms with proteolytic and lipolytic potential recently described by our group (Ribeiro Júnior et al., 2018b). Thus, even if pasteurization is applied following bacto-fugation, these microorganisms can survive.

Additionally, because counts of psychrotrophs in raw milk were high ($>10^6$), it is likely that the quality of pasteurized bacto-fuged milk was impaired by the large amounts of thermostable microbial proteases and lipases remaining in the raw milk (Cousin, 1982). The cheese factory itself has reported problems with late proteolysis over the life of the products, even without expressive isolation of proteolytic bacteria in the final product.

As an alternative to minimize the residual deteriorating effect of these enzymes, some researchers have suggested a process of gravitational separation of microorganisms and microbial spores of milk (Caplan et al., 2013; Geer and Barbano, 2014). Because gravitational separation does not involve cellular lysis, there is no release of the enzymes from the interior of the bacteria. During bacto-fugation, preheating and the application of gravitational force may promote cellular lysis (Kosikowski and Fox, 1968).

Bacillus licheniformis was isolated among the psychrotrophs in raw and preheated milk but was not identified in bacto-fuged milk. *Bacillus licheniformis* is a major thermotolerant (Ribeiro Júnior et al., 2018b), spore-forming species (Ribeiro Júnior et al., 2018c) in milk in Brazil and other countries (Reginensi et al., 2011; Yuan et al., 2012; Martinez et al., 2017). Thus, bacto-fugation appears to be effective in reducing *B. licheniformis*, which is important for the quality of processed milk. However, as other spore-forming bacteria remained following bacto-fugation (*L. fusiformis* and *B. invictae*), we cannot state that there is no risk of isolation of *B. licheniformis* in bacto-fuged milk.

The process of preheating raw milk before bacto-fugation is sufficient to considerably reduce the count and diversity of raw milk psychrotrophs. Bacto-fugation apparently has no effect on any particular group or genus of psychrotrophs. However, it was able to further reduce bacterial counts compared with preheating alone. Thus, bacto-fugation can be used to reduce the psychrotrophic bacterial counts of raw milk, especially before refrigerated storage in dairy factories, minimizing bacterial multiplication and the production of thermostable deteriorating enzymes.

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