



Efficient removal of spores from skim milk using cold microfiltration: Spore size and surface property considerations

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ABSTRACT

Bacterial spores present in milk can cause quality and shelf-life issues for dairy products. The objectives of this study were to evaluate the effectiveness of microfiltration (MF) in removing *Bacillus licheniformis* and *Geobacillus* sp. spores from skim milk using membranes with pore sizes of 1.4 and 1.2 μm , and to investigate the role of spore surface properties in MF removal. Cell sizes were determined by scanning electron microscopy, surface charge by zeta potential analysis, and surface hydrophobicity by contact angle measurements. Commercially pasteurized skim milk was inoculated with a spore suspension at about 10^6 cfu/mL, and then processed by MF using ceramic membranes at 6°C, a cross-flow velocity of 4.1 m/s, and transmembrane pressure of 69 to 74 kPa. Total aerobic plate and spore counts in the milk were determined before and after MF. All processing runs and surface and product analyses were conducted in triplicate, and data were analyzed statistically. For the same strain, spores were shorter and wider than vegetative cells, averaging 1.37 to 1.59 μm in length and 0.64 to 0.81 μm in width. Reduction of *B. licheniformis* spores significantly increased with a reduction in MF pore size, from 2.17 log for 1.4- μm pore size, to 4.57 log for 1.2- μm pore size. Both pore sizes resulted in almost complete removal of *Geobacillus* sp. spores. All spores and the ceramic membrane had a negative surface charge at milk pH, indicating an electrostatic repulsion between them. *Bacillus licheniformis* spores were hydrophilic, whereas *Geobacillus* sp. spores were hydrophobic. Consequently, *Geobacillus* sp. spores had a tendency to cluster in skim milk, preventing their passage even through the 1.4- μm MF membrane, whereas some *B. licheniformis* spores could still pass through a 1.2- μm membrane. This study demonstrates that efficient removal of spores from skim milk by cold MF may require a smaller membrane pore size than required for removal of vegetative cells of the same spe-

cies, and that cell surface properties may interfere with the outcome of filtration as would be anticipated based on size alone.

Key words: microfiltration, spore removal, skim milk

INTRODUCTION

The presence of spore-forming bacteria in milk is a critically important issue in the dairy industry. Bacterial endospores can survive in harsh environmental conditions such as high heat, low pH, desiccation, or cleaning and sanitizing chemicals (Scheldeman et al., 2006; Burgess et al., 2010; Ranieri and Boor, 2010). Compared with vegetative cells, spores have also been found to attach more readily to stainless steel, leading to the formation of biofilms that can promote bacterial contamination within dairy processing plants (Rönner et al., 1990; Parkar et al., 2001; Palmer et al., 2010). Spores present in final products can germinate and produce enzymes that decrease dairy product quality and shelf life, causing significant economic losses (Crielly et al., 1994; Postollec et al., 2012; Lücking et al., 2013). Additionally, some sporeformers such as *Bacillus cereus* and *Bacillus subtilis* can produce toxins that are responsible for food poisoning (De Jonghe et al., 2010). Thermophilic *Geobacillus* spp. and *Anoxybacillus* spp. are other sporeformers of importance to the dairy industry, as they are commonly isolated from dairy powders and evaporated milk (Rückert et al., 2004; Scheldeman et al., 2006; Watterson et al., 2014). Removal of all these spores from milk can result in significant improvements in quality and shelf life of dairy products.

Microfiltration (MF) is an effective nonthermal method for removing bacteria and somatic cells from skim milk, while maintaining milk quality virtually unchanged. Microfiltration is used in the industry to produce extended-shelf-life milk, and as a potential pretreatment of skim milk intended for the manufacture of dairy powders or cheese. Previous studies reported average reductions of vegetative bacteria from 3 to 6 log cfu/mL when using MF membranes with a pore size of 1.4 μm for microbial removal from skim milk (Elwell and Barbano, 2006; Hoffmann et al., 2006; Schmidt

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et al., 2012). However, Schmidt et al. (2012) reported that two-thirds of commercial extended-shelf-life milk produced in Germany, Austria, and Switzerland using MF followed by pasteurization underwent spoilage by *B. cereus* and *Paenibacillus* spp., suggesting that although MF membranes of 1.4- μm pore size can remove vegetative cells, they may not sufficiently eliminate spores, which may be smaller than vegetative cells of the same species. Spores of *Bacillus* spp. were reported to range from 1.07 to 1.74 μm in length and 0.48 to 0.98 μm in diameter (Leuschner et al., 1999; Carrera et al., 2007), and thus some may pass through a 1.4- μm membrane. Reducing the membrane pore size may improve spore reduction. For example, Tomasula et al. (2011) reported average 4.50- and 5.91-log reductions of *Bacillus anthracis* (Sterne) from skim milk using 1.4- and 0.8- μm ceramic membranes, respectively, but the 0.8- μm membrane led to significant protein reduction in the permeate, suggesting that this pore size may be too small for maintaining the skim milk composition.

Factors other than size may also play a role in bacterial spore removal by MF. For example, surface structural elements, such as proteins or exosporium, or surface properties, such as hydrophobicity and electrostatic potential, can influence the interactions between cells, as well as between cells and the membrane surface (Seale et al., 2008; Palmer et al., 2010; Feng et al., 2015). Temperature is a critical parameter in membrane filtration, and its effect on bacteria removal needs to be considered. In the dairy industry, bacterial removal by MF is typically conducted under warm conditions, at temperatures between 50 and 55°C. Although conducting MF warm can maximize permeate flux, these conditions also allow for optimum growth of thermophilic (*Geobacillus* spp. and *Anoxybacillus* spp.) or facultative thermophilic (certain strains of *Bacillus* spp.) sporeformers (Burgess et al., 2010). Warm MF may thus promote the growth of sporeformers within the membrane and recirculation loop of the MF system, which can result in microbial fouling of the MF membranes (Kochkodan et al., 2006; Tang et al., 2009; Anand et al., 2014). This could then lead to formation of highly resistant biofilms, which can serve as a reservoir of bacteria and propagate recontamination in the system and in the final product (Flint et al., 2001; Parkar et al., 2001). Low-temperature MF has therefore been proposed as a solution to reduce the risk of bacterial growth during membrane processing (Fritsch and Moraru, 2008; Walkling-Ribeiro et al., 2011).

Studies focusing on the specific removal of spores relevant to dairy product quality using MF are very limited. Elwell and Barbano (2006) and Fritsch and Moraru (2008) detected no spores in skim milk subjected to MF with a 1.4- μm membrane, under either warm

(the former) or cold (the latter) conditions. However, in these studies, the initial raw milk had a very low spore count, and thus the results are inconclusive regarding the full potential of MF for spore retention. To address this gap, the main objective of the current study was to evaluate the effectiveness of 1.2- and 1.4- μm pore size membranes on the removal of vegetative cells and bacterial spores from skim milk by cold MF. Additionally, the cell size and surface properties of the challenge microorganisms were determined and their impact on physical removal by MF was evaluated. As challenge organisms, vegetative cells and spores of *B. licheniformis* and *Geobacillus* sp. were used. The 2 microorganisms are recognized culprits for dairy product spoilage (Burgess et al., 2010; Lücking et al., 2013; Watterson et al., 2014). The findings of this study can be used to develop more efficient MF processes for the removal of spores from milk, leading to the production of dairy products with improved quality and shelf life.

MATERIALS AND METHODS

Production of Bacterial Spore Suspensions

The procedure for producing the spore suspensions was adapted from Gaillard et al. (1998), with minor modifications, and is described below.

Inoculation and Plating. Isolated cultures of *B. licheniformis* (FSL strain F4-0073) and *Geobacillus* sp. (FSL strain W8-0032) were obtained from the Cornell Milk Quality Improvement Program (Department of Food Science, Ithaca, NY). Five milliliters of brain heart infusion broth (BBL, Becton Dickinson and Co., Franklin Lakes, NJ) was inoculated with cultures of each study species and incubated overnight (*B. licheniformis* at 32°C; *Geobacillus* sp. at 55°C). A volume of 250 μL of culture was plated onto sporulation agar. *Bacillus licheniformis* was sporulated on AK#2 agar (BBL, Becton Dickinson and Co.) at 32°C for an average of 10 d, and *Geobacillus* sp. was sporulated on tryptone glucose extract agar (Difco, Becton Dickinson and Co.) with 10 mg/L manganese sulfate at 55°C for an average of 21 d. Sporulation was monitored during incubation by staining with 0.5% malachite green solution. Spores were harvested when staining showed sporulation of $\geq 95\%$.

Harvesting. To harvest spores, 15 mL of sterile phosphate buffer (Weber Scientific, Hamilton, NJ) was added to the agar surface. Cells were scraped off using a sterile loop, and the resulting suspension was collected in a sterile centrifuge tube. Suspensions were centrifuged at 4°C using a Beckman Avanti J-25 centrifuge (Beckman Coulter, Fullerton, CA). Centrifugation was conducted at $11,086 \times g$ for 10 min for *B.*

licheniformis, and $17,510 \times g$ for 15 min for *Geobacillus* sp. The supernatant was drained and resuspended in a 50% ethanol solution for up to 3 d at 4 to 6°C. Following storage in ethanol, suspensions were recentrifuged and the supernatant was drained. Spores were washed 3 times and resuspended in sterile phosphate buffer. Final suspensions were stained with 0.5% malachite green solution and checked microscopically to ensure that no germination occurred during harvesting. To determine suspension concentration (cfu/mL), an aerobic plate count was performed with serial dilutions in 0.1% peptone (Difco, Becton Dickinson and Co.) spread plating in duplicate on brain heart infusion agar at 32°C (*B. licheniformis*) or 55°C (*Geobacillus* sp.) for 48 h (Frank and Yousef, 2004). Final suspensions were stored at 4 to 6°C until use.

Physical Characterization of Bacterial Spores

Imaging by Scanning Electron Microscopy. Samples were prepared for scanning electron microscopy imaging according to Feng et al. (2014). A piece of glass coverslip was broken into pieces of $\sim 0.5\text{-cm}^2$ and thoroughly rinsed in acetone, ethanol, and MilliQ water (Millipore Sigma, Burlington, MA). Rinsing was followed by 1 h of sonication in fresh MilliQ water. To fix the samples onto the substrate, 10 μL of spore or vegetative cell suspension was pipetted onto a piece of clean coverslip, air-dried under a biosafety hood for 1 h, and then fixed with 2.5% (wt/vol) glutaraldehyde in 0.05 M sodium cacodylate buffer at 4°C for 2 h. After this, samples were rinsed 3 times in cacodylate buffer for 5 min per rinse, and then treated with 1% (wt/vol) osmium tetroxide in cacodylate buffer for 1 h. Subsequently, samples were rinsed in cacodylate buffer 3 times and sequentially dehydrated in 10-min steps using 25% (vol/vol), 50%, 70%, 95%, and 100% ethanol solutions, respectively. After dehydration, samples were dried to critical point with carbon dioxide. The dried surfaces were mounted onto scanning electron microscopy stubs and coated with evaporated carbon. The scanning electron microscopy imaging of spores and vegetative cells was conducted with a Zeiss LEO 1550 field emission scanning electron microscope (Carl Zeiss Microscopy LLC, Hamburg, Germany), at a voltage of 3 kV. Micrographs were acquired using SmartSEM software (Carl Zeiss Microscopy LLC).

The dimensions of vegetative cells and spores were determined on scanning electron micrographs using ImageJ analytical software (W. S. Rasband; National Institutes of Health, Bethesda, MD). Average dimensions were calculated using sets of 9 to 19 cells. A Gaussian distribution of cell size was developed based on the average length and range for each strain, and

used to estimate the probability of cells to be larger or smaller than the membrane pore size (1.2 and 1.4 μm , respectively). The detailed methodology for these calculations can be found in Supplemental File S1 (<https://doi.org/10.3168/jds.2018-14888>).

Contact Angle Measurement of Bacterial Spores and the Ceramic Membrane. Contact angles of water, glycerol, and diiodomethane on lawns of bacterial spores and vegetative cells were determined by the sessile drop method with a Rame-Hart 500 goniometer (Rame-Hart Inc., Succasunna, NJ), as described previously by Feng et al. (2014). Contact angles of the active layer of a cleaned, unused ceramic MF membrane were also measured.

To prepare the bacterial lawns, 30 mL of spore or vegetative cell suspension was centrifuged for 10 min at $1,789 \times g$ and 4°C using a Hettich Universal 32R centrifuge (Hettich Lab, Tuttlingen, Germany). The resulting pellet was resuspended with Butterfield phosphate buffer (BPB) formulated to simulate the pH and ionic strength of milk (pH = 6.8, ionic strength = 0.8 M, at 6°C). The suspension was centrifuged again under the same conditions to ensure total replacement of the initial suspension medium by a milk-mimicking BPB. After the supernatant was decanted, the pellet was resuspended with 15 mL of fresh BPB to double the concentration. Two 15-mL tubes of the resultant suspension were filtered through a 0.22- μm polyether-sulfone membrane (47 mm diameter, Millipore Express Plus; Merck Millipore Ltd., Billerica, MA) using a magnetic filtration funnel, leading to the formation of a bacterial lawn. The lawn was dried and mounted onto a glass slide before contact angle measurement.

Measurement of Zeta Potential. Following the procedure described by Feng et al. (2014), the zeta potential of spores and vegetative cells was measured in BPB (pH 6.8, ionic strength = 0.8 M, at 6°C) using a Malvern Zetasizer nano-ZS with disposable folded capillary cells (Malvern Instruments, Malvern, UK). To measure the surface zeta potential of the ceramic filtration membrane, an unused membrane was cut to the size of the plastic stage on which it was to be mounted. Membrane sections were cleaned and mounted via double-sided tape to the stage. The membrane's active surface was oriented toward the electrodes, and the stage was placed between the electrodes of a surface zeta potential cell (Malvern Instruments). The probe was submerged in 1.0 mL of tracer particle suspension (Malvern Instruments) contained in a plastic cuvette. Measurements of electrophoretic mobility of tracer particles at 125, 250, 375, 500, and 625 μm from the planar surface of the MF membrane surface were taken at 6°C. The magnitude of particle electrophoresis and electro-osmosis generated by the surface charge were used to

derive the zeta potential at the wall surface following the methods of Corbett et al. (2012). Electrophoretic measurements were averages of at least 20 internal repetitions; triplicate measurements were performed and averaged to calculate the apparent zeta potential values at each distance from the surface. Measurements of electrophoretic velocity of tracer particles were repeated 3 times, with averaging of 30 internal measurements for each repeat, at 1,000 μm from the surface.

Gibbs Free Energy Calculations. The change in Gibbs free energy was used as a thermodynamic indicator of cell–cell and cell–surface interactions, which considers acid–base and van der Waals forces. The method for calculating the Gibbs free surface energy of aggregation (ΔG_{bwb}) and surface energy of attachment (ΔG_{bws}) from the contact angle measurements, as well as the individual surface energy components can be found in Supplemental File S1 (Table S1; <https://doi.org/10.3168/jds.2018-14888>). It should be noted that the physico-chemical constants used to calculate the Gibbs free energy were measured at 20°C, and thus the calculated values would hold for this temperature. To accurately account for the effect of temperature on hydrophobic interactions, the Gibbs free energy of aggregation was also calculated at 6°C by extrapolating the values of surface tension components (γ_{LW} and γ_{AB}) at 6°C from the study of Zdziennicka et al. (2017).

MF Experiments

A pilot-scale MF unit consisting of a 50-L feed tank with a variable speed centrifugal pump, heat exchanger, and membrane housing, equipped with Isoflux tubular ceramic membranes of Tami design (GEA Filtration, Hudson, WI) was used. Membranes were 1,200 mm long and had an outer diameter of 25 mm, 23 internal channels of 3.5-mm hydraulic diameter each, and a total membrane area of 0.35 m^2 . Two new membranes with pore sizes of 1.4 and 1.2 μm were used throughout the study.

An inline temperature probe was used to monitor the temperature, and pressure gauges were used to measure feed inlet (P_i) and feed outlet (P_o) pressure. The transmembrane pressure (**TMP**) was calculated using the equation:

$$\text{TMP} = \frac{(P_i + P_o)}{2} - P_p,$$

where P_i is feed inlet pressure, P_o is feed outlet pressure, and P_p is permeate pressure.

Before each experimental run, the MF unit, previously chemically cleaned, was sterilized with hot water

(80°C) for 30 min. Pasteurized skim milk, obtained from the Cornell Dairy Plant (Ithaca, NY), was stored at 6°C until the MF run. The *B. licheniformis* experiments were conducted on 3 separate days each for the 1.2- and 1.4- μm pore sizes during June and July 2015. *Geobacillus* sp. experiments were similarly conducted on 3 days during September and October 2015.

A volume of 200 to 400 mL of spore suspension of known concentration was centrifuged, the phosphate buffer decanted, and a spore pellet recovered. The spore pellet was then resuspended in aseptically collected skim milk, and this suspension was then used for direct inoculation of the skim milk in the feed tank of the MF unit.

Microfiltration of the inoculated skim milk was conducted at 6°C with a cross-flow velocity of 4.14 ± 0.01 m/s. The TMP averaged 73.11 ± 5.93 and 69.87 ± 1.62 kPa for the 1.4- and 1.2- μm processing runs, respectively. These MF parameters were selected to minimize fouling, based on the study by Fritsch and Moraru (2008), because excessive fouling could have caused inflated spore removal counts unrepresentative of true membrane selectivity. For the same reason, MF processing was only conducted for a short duration (18 to 19 min), sufficient to collect enough permeate for analysis without significant fouling of the membrane. Permeate samples were collected aseptically using an inline sampling port and sterile sampling assembly (Quality Management Inc., Oakdale, MN). The collected sample bags were immediately placed on ice or in refrigerated storage until analysis, which was conducted within 2 h of collection. The MF experiments were conducted in triplicate for each spore species and each membrane pore size.

Following each MF run, membranes were chemically cleaned starting with a 10-min reverse osmosis (**RO**) water rinse. This was followed by a 30-min alkaline clean using 17.5 mL/L of Ultrasil-25 (Ecolab, St. Paul, MN) at 80°C, followed by a second 10-min RO water rinse, a 20-min acid cleaning using 5 mL/L of HNO_3 at 50°C, and a final RO water rinse. Water flux measurements were conducted before and after MF runs to monitor cleaning efficacy and any changes in membrane performance over time.

Microbiological Analyses

Preinoculated pasteurized milk and MF permeate samples were tested for mesophilic spore counts for *B. licheniformis* challenge studies and thermophilic spore counts for the *Geobacillus* sp. challenge studies, using the methodology of Frank and Yousef (2004). Briefly, to determine the spore counts, a spore pasteurization step was completed as follows: samples were heated and

held at 80°C for 12 min, and then cooled to 6°C before sample dilution and plating. This step is necessary to destroy vegetative cells in the sample and germinates spores, ensuring a final count of only bacterial spores. Serial dilutions were spread plated in duplicate on brain heart infusion agar at for 48 h (mesophilic at 32°C; thermophilic at 55°C). All plates were counted and results expressed in log colony-forming units per milliliter.

Composition Analyses

The chemical composition of the milk samples was determined using a Milkoscan Minor (Foss, Hillerød, Denmark) in duplicate.

Statistical Analyses

Data were analyzed using RStudio statistical software (version 3.2.2, RStudio Inc., Boston, MA). Statistical differences among observed means were determined using an unpaired *t*-test with a significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

MF with Different Pore Sizes: Effect on Spore Removal, Milk Quality, and Flux

Microfiltration using the 1.2- μm membrane resulted in a significantly ($P < 0.005$) greater reduction of *B. licheniformis* spores ($4.57 \log \pm 0.09$) than the 1.4- μm membrane ($2.17 \log \pm 0.64$; Table 1). For *Geobacillus* sp., spore counts in the MF milk were below the detection limit, indicating near-complete reduction ($>6 \log$) for MF with both pore sizes. Although the nominal pore size of the 1.2- μm membrane was considerably larger than the size of all milk components (excluding the milk fat globules), this membrane also resulted in a greater retention of milk components than the 1.4- μm membrane, as shown in Table 2. Although the largest percentage reduction of all components occurred for milk fat, the initial fat content of the milk was very low

($\sim 0.22\%$), because the MF was conducted using skim milk. Lactose content was only minimally affected by MF, because lactose is a small, soluble molecule and thus not retained by MF membranes. Most substantial was the reduction of milk proteins in the MF permeate, with reductions of $\sim 4\%$ for the 1.4- μm membrane and $\sim 10\%$ for the 1.2- μm membrane. This is most likely due to retention of casein micelles, which was enhanced by the smaller pore size. Brans et al. (2004) suggested that the bridging of casein micelles can lead to partial pore blocking of MF membranes. Tan et al. (2014) also found that casein micelles were a major cause of fouling in cold MF using a 1.4- μm membrane, because of micelle interaction and binding to the membrane surface. Minimizing protein retention is very important for the implementation of MF in the dairy industry, because protein is an important component of skim milk, from both a nutritional and an economical point of view. Optimization of processing parameters for 1.2- μm MF can identify the processing parameters that appropriately balance maximum bacterial removal, minimum protein retention, and maximum flux. Additionally, the MF retentate, which has both a high protein content and a high bacterial count, can be separately processed using high heat and later recombined with the permeate, as described by Holm et al. (1986).

The greater retention of these milk components was reflected in the lower flux values and slightly higher flux drop over time obtained for the 1.2- μm membrane compared with the 1.4- μm membrane (Figure 1). In Figure 1, data were grouped for the 2 types of spores, because some irreversible membrane fouling may have occurred with time and because slight seasonal changes in milk composition could have affected the filterability of the milk. Although the flux values for the 2 membrane pore sizes did not differ statistically within a spore species due to the large variability of the data, similar trends were observed for both spore challenges. These data align well with the changes in composition reported for the *Geobacillus* sp. challenge reported above.

Nonetheless, the most remarkable result in this study was the much higher reduction of *B. licheniformis*

Table 1. Average reduction (\pm SD) of bacterial spores by microfiltration (MF) processing ($n = 3$)

Challenge study	Initial load (log cfu/mL)	After MF (log cfu/mL)	Total log reduction (log cfu/mL)
<i>Bacillus licheniformis</i>			
1.4- μm MF membrane	6.11 ± 0.53	3.94 ± 0.13	2.17 ± 0.64^a
1.2- μm MF membrane	6.98 ± 0.08	2.41 ± 0.15	4.57 ± 0.09^b
<i>Geobacillus</i> sp.			
1.4- μm MF membrane	6.56 ± 0.29	ND ¹	$>6 \log$
1.2- μm MF membrane	6.38 ± 0.24	ND	$>6 \log$

^{a,b}Means within a column with different superscripts differ significantly ($P < 0.05$).

¹Not detected (counts were below the detection limit of 25 cfu/plate).

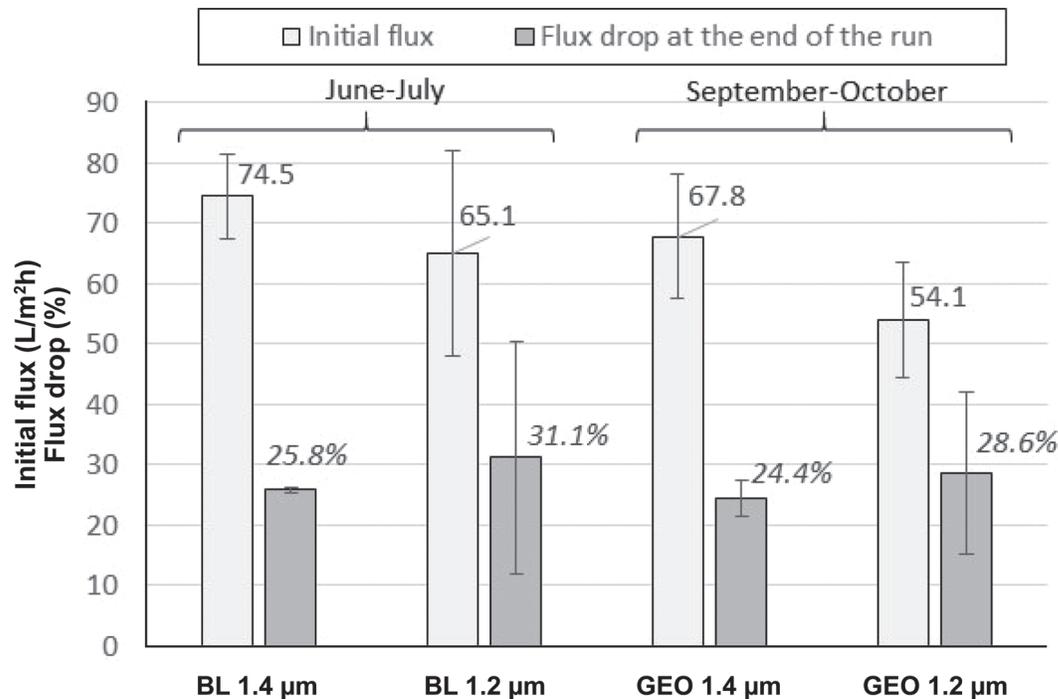


Figure 1. Permeate flux data for the cold microfiltration (MF) of skim milk inoculated with *Bacillus licheniformis* (BL) and *Geobacillus* sp. (GEO) spores. The duration of an experimental MF run was 18 to 19 min. Data points represent averages of 3 experimental replicates, with the exception of the *B. licheniformis* 1.4 µm challenge, for which only 2 reliable flux data sets were available. Error bars represent means \pm 1 SD.

spores by the 1.2-µm membrane than by the 1.4-µm membrane, and the remainder of the paper will focus on the reasons for these differences.

Effect on Spore Size on Removal by MF: Size Matters

Under scanning electron microscopy, the vegetative cells of both bacterial strains appeared as long rods, characteristic of the genera studied (Figure 2). Vegetative cell length exceeded 2 µm and width was less than 0.5 µm for both species (Table 3). Although *B. licheniformis* vegetative cells were shorter in length than *Geobacillus* sp. cells, they occasionally formed

long chains, reaching 20 µm or more (Figure 1), as has been previously reported (Herben et al., 1990). Vegetative cells of *Geobacillus* sp. formed either clusters or short chains consisting of 2 to 3 cells (Figure 2). For both species, spores were shorter and wider than their respective vegetative cells, averaging 1.37 to 1.59 µm in length and 0.64 to 0.81 µm in width (Table 3), which is consistent with previously reported spore sizes of *Bacillus* species (Leuschner et al., 1999; Carrera et al., 2007).

Geobacillus sp. spores (1.59 µm in length and 0.81 µm in width) were, in principle, large enough to be retained by both membranes. At 1.37 µm long and 0.64 µm wide, *B. licheniformis* spores should be largely

Table 2. Changes in skim milk composition after microfiltration (MF) with 1.4- and 1.2-µm membranes following *Geobacillus* sp. spore challenge MF runs

Sample	Component			
	TS (%)	Protein (%)	Lactose (%)	Fat (%)
Initial skim milk (control)	9.26 \pm 0.08 ^a	3.36 \pm 0.04 ^a	4.63 \pm 0.04 ^a	0.22 \pm 0.01 ^a
Permeate: 1.4 µm	8.96 \pm 0.07 ^b	3.21 \pm 0.02 ^b	4.59 \pm 0.03 ^a	0.15 \pm 0.01 ^b
Change ² (%)	-3	-4	-1	-32
Permeate: 1.2 µm	8.73 \pm 0.05 ^c	3.03 \pm 0.06 ^c	4.58 \pm 0.03 ^a	0.12 \pm 0.02 ^c
Change (%)	-6	-10	-1	-45

^{a-c}Means within a column with different superscripts differ significantly ($P < 0.05$).

¹Values represent averages of 3 MF runs \pm 1 SD.

²Compared with control.

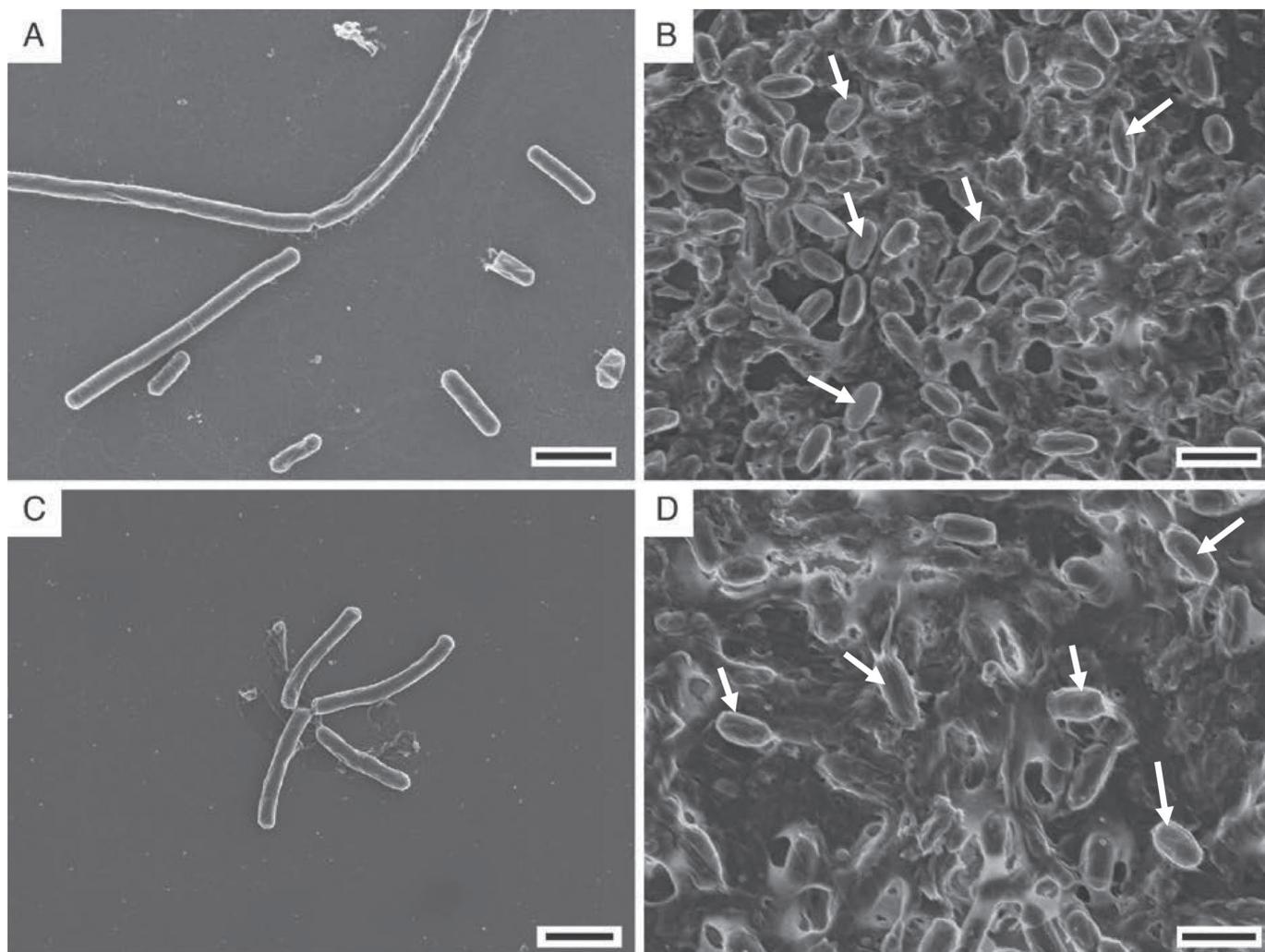


Figure 2. Scanning electron micrographs of vegetative cells and spores, respectively, of *Bacillus licheniformis* (A, B) and *Geobacillus* sp. (C, D). The small white arrows in the right panels indicate some examples of spores. Scale bar = 2 μ m.

retained by the 1.2- μ m membrane but may be able to pass through the 1.4- μ m membrane in any orientation. The fact that a significant retention of *B. licheniformis* occurred even with the larger pore size membrane can be explained by (1) membrane fouling; (2) the variability in spore size; or (3) interactions between spores or

between spores and the membrane material. Membrane fouling may have played a role, but it was minimal for this membrane pore size (see Figure 1, Table 2) due to carefully chosen processing parameters, as well as the short run time. Although the short run time does not reflect standard industry practices (minutes vs. hours), it was necessary for this particular study to determine the full extent to which spores may be able to pass through an unfouled membrane. As fouling develops, increased spore retention is expected because of blocked or constricted membrane pores, thereby improving overall spore removal in the permeate. The results presented here represent the “worst-case scenario” for spore removal by a largely unfouled membrane. Overall spore removal may actually be higher in industrial applications (which would use much longer run times) due to fouling, although fouling will also negatively

Table 3. Measured vegetative cell and spore sizes of *Bacillus licheniformis* and *Geobacillus* sp.¹

Bacterial species	Long axis (μ m)	Short axis (μ m)
<i>B. licheniformis</i>		
Vegetative cell (n = 12)	2.24 \pm 0.26	0.47 \pm 0.02
Spore (n = 19)	1.37 \pm 0.03	0.64 \pm 0.01
<i>Geobacillus</i> sp.		
Vegetative cell (n = 9)	2.79 \pm 0.21	0.43 \pm 0.02
Spore (n = 16)	1.59 \pm 0.05	0.81 \pm 0.02

¹Values represent mean \pm SE (n = 9–19 cells).

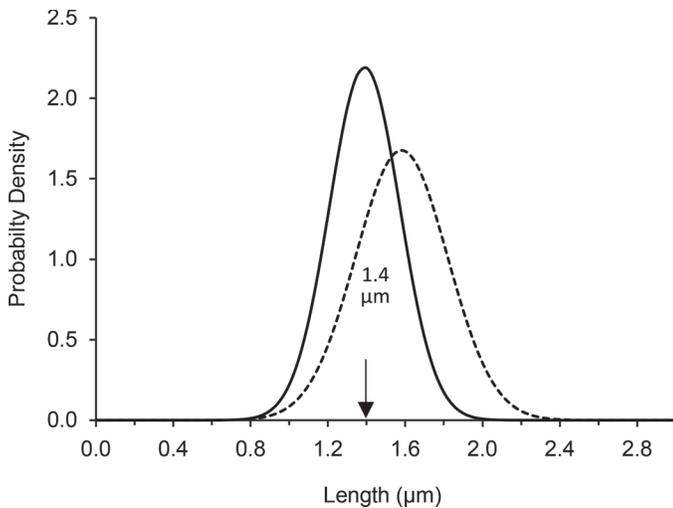


Figure 3. Gaussian distribution of spore lengths (μm) for *Bacillus licheniformis* (solid line) and *Geobacillus* sp. (dashed line) generated based on measured cell sizes. The arrow indicates $1.4 \mu\text{m}$, which is the nominal pore size average of the commercial microfiltration membrane.

affect protein permeation, decreasing the final protein content in the MF milk.

Because fouling was minimal in this study, we cannot explain why only about 0.7% of the initial spores passed into the permeate. Thus, we will focus on the other 2 possible causes.

As discussed before, size is a crucial factor in membrane filtration. The values in Table 3 were used to generate a Gaussian distribution for spore size (Figure 3), which was then used to calculate the probability that spore lengths were less than 1.4 and $1.2 \mu\text{m}$ for each strain. Based on the generated distributions, *B. licheniformis* spores have a 58.7% probability of being shorter than $1.4 \mu\text{m}$, and only a 10.7% probability

of being shorter than $1.2 \mu\text{m}$. *Geobacillus* sp. spores are less likely to pass through $1.4\text{-}\mu\text{m}$ pores (15.2% chance), and even less likely to pass through $1.2\text{-}\mu\text{m}$ pores (1.6% chance). An additional factor that needs to be considered is the distribution of membrane pore size (data not available). Nominal pore sizes listed by the manufacturer for these membranes represent average values, but it is known that membranes typically have a pore size distribution (Saboyainsta and Maubois, 2000). Although current ceramic membranes have a relatively narrow pore size distribution, it is to be expected that pores both larger and smaller than the nominal pore size exist for both types of membranes. Some variability in the membrane structure can be observed in scanning electron micrographs of ceramic membranes similar to those used in the current study in the papers by Fritsch and Moraru (2008) and Tan et al. (2014). Therefore, shorter *Geobacillus* sp. spores could have passed through the larger pores of the $1.4\text{-}\mu\text{m}$ membrane, which was not the case experimentally. Therefore, possible interactions between spores or between spores and the membrane material also need to be considered.

Contribution of Spore–Spore and Spore–Membrane Interaction Forces

Zeta potential and surface hydrophobicity for the microbial cells and the membrane material were determined and used to examine the possibility of interactions between spores and the MF membrane (Table 4).

Both the microbial cells (vegetative cells and spores) and the membrane were found to be negatively charged at an ionic strength equal to that of milk, which is consistent with previous reports (Ankolekar and Labbé,

Table 4. Zeta potential, contact angle measurements, and Gibbs free energy values for an unused ceramic membrane and *Bacillus licheniformis* and *Geobacillus* sp. cell suspensions¹

Species and material	Zeta potential at 20°C (mV)	Contact angle (θ ; $^\circ$) at 20°C			Gibbs free energy ² (mJ/m^2)			
		θ_{Water}	θ_{Glycerol}	$\theta_{\text{Diiodomethane}}$	At 20°C		At 6°C ³	
					ΔG_{bwb}	ΔG_{bws}	ΔG_{bwb}	ΔG_{bws}
<i>B. licheniformis</i>								
Vegetative cells	-15.62 ± 0.13	36.63 ± 1.24	76.20 ± 2.41	80.62 ± 2.06	83.37	59.02	81.65	59.63
Spores	-14.71 ± 0.73	38.76 ± 2.15	67.00 ± 4.08	<10	27.72	39.25	32.38	40.03
<i>Geobacillus</i> sp.								
Vegetative cells	-5.92 ± 0.04	33.28 ± 1.42	93.11 ± 1.93	27.51 ± 5.11	14.99	57.01	15.55	56.10
Spores	-10.39 ± 0.42	80.56 ± 4.05	78.71 ± 8.29	50.74 ± 2.78	-35.94	4.19	-30.26	6.77
Ceramic membrane	-45.57 ± 3.28	<10	27.67 ± 1.78	<10	NA ⁴			

¹Values represent mean \pm SE ($n = 3$ for all measurements).

²Gibbs free energy values of aggregation (ΔG_{bwb}) and attachment (ΔG_{bws}) were calculated at both the temperature of zeta potential and contact angle measurements (20°C) and the temperature of the microfiltration runs (6°C).

³The surface tension components (γ_{LW} and γ_{AB}) at 6°C were calculated using data extrapolated from Zdziennicka et al. (2017).

⁴NA = not applicable.

2010; Palmer et al., 2010). It should also be noted that although the zeta potential for *B. licheniformis* did not change drastically upon sporulation, the charge of *Geobacillus* sp. spores was twice that of their respective vegetative cells. The ceramic membrane (new, unused material) had a negative charge between 3 and 4.5 times higher than any of the bacterial cells, and thus the membrane should repel both the vegetative cells and spores of both species. Therefore, it is unlikely these slight differences in zeta potential have a significant effect during MF.

Hydrophobicity has been extensively studied in regard to bacterial cell interactions. Spores have generally been found to be more hydrophobic than vegetative cells, which is thought to be a major factor in the stronger attachment of spores—compared with vegetative cells—to stainless steel (Doyle et al., 1984; Rönner et al., 1990; Simmonds et al., 2003). When measured by water contact angle (WCA), bacterial surfaces and cell lawns have been characterized as either very hydrophilic (WCA <40°), very hydrophobic (WCA >90°), or in between (Mozes and Rouxhet, 1987; Simmonds et al., 2003). In this work, *B. licheniformis* remained very hydrophilic upon sporulation, with a WCA of $36.63 \pm 1.24^\circ$ and $38.76 \pm 2.15^\circ$ for vegetative cell and spores, respectively. *Geobacillus* sp., on the other hand, had very hydrophilic vegetative cells (WCA = $33.28 \pm 1.42^\circ$) but became significantly ($P < 0.05$) more hydrophobic in spore form (WCA = $80.56 \pm 4.05^\circ$). Although previous researchers found spores within the genus of *Geobacillus* to be hydrophilic (Seale et al., 2008), others have noted that it is possible for bacterial strains within the same genus to range from hydrophilic to hydrophobic, and that hydrophobicity may change depending on growth medium, temperature, and method for measurement (Mozes and Rouxhet, 1987; van der Mei et al., 1998; Simmonds et al., 2003). The new (unused) membrane material was very hydrophilic. However, Tan et al. (2014) reported that although ceramic membranes begin as hydrophilic, adsorption and deposition of milk proteins during MF processing leads to a more hydrophobic membrane surface. The implication is that there might be differences in cell removal by the membrane even over the course of the MF process, as fouling develops.

In addition to this assessment of surface hydrophobicity, we estimated the Gibbs free energy of aggregation (ΔG_{bwb}) and attachment (ΔG_{bws}). These calculations are based on contact angle measurements performed with 3 liquids that differ in polarity and water solubility. Using this approach, the van der Waals and acid-base components of the interaction forces between cells and between cells and the membrane surface can be accounted for, resulting in a more complete picture

of their thermodynamic interactions. In an aqueous environment such as milk, hydrophobic particles tend to aggregate, resulting in a $\Delta G_{\text{bwb}} < 0$, whereas hydrophilic particles ($\Delta G_{\text{bwb}} > 0$) favor water and have no tendency to reduce surface area through clustering. Similarly, a negative ΔG_{bws} would indicate thermodynamic attraction to a surface, whereas a positive ΔG_{bws} indicates no thermodynamic attraction. The Gibbs free surface energies for the unused ceramic membrane material, as well as the *B. licheniformis* and *Geobacillus* sp. cell suspensions, are shown in Table 4. Most of the results are consistent with those obtained from the WCA measurements alone, and therefore will not be discussed. The most notable information is that, based on the value of the Gibbs free energy of aggregation the hydrophobic *Geobacillus* sp. spores tend to cluster in an aqueous environment of milk, at both 20°C ($\Delta G_{\text{bwb}} = -35.94 \text{ mJ/m}^2$) and at 6°C, the temperature of the MF runs ($\Delta G_{\text{bwb}} = -30.26 \text{ mJ/m}^2$). The larger size of the clusters compared with individual cells would prevent their passage through the MF membrane, which better justifies the full retention of these spores by the 1.4- μm membrane.

CONCLUSIONS

These results demonstrated that although vegetative bacterial cells are effectively removed from skim milk using 1.4- μm MF membranes, spores may require a smaller pore size for effective removal because of their smaller size compared with vegetative cells. In a challenge study, physical removal of *B. licheniformis* spores was significantly increased when MF pore size was decreased from 1.4 to 1.2 μm . An evaluation of surface interactive elements was useful in identifying the thermodynamic tendency of *Geobacillus* sp. spores to cluster, which helped their removal by MF, even when using a 1.4- μm membrane. Conversely, some *B. licheniformis* were still able to pass through the 1.2- μm membrane. A negative consequence of the reduced pore size was a greater reduction of milk protein concentration in the MF milk by the smaller pore size membrane. This was attributed to increased retention of casein micelles by the smaller membrane, which indicates the need to optimize processing parameters for the MF process, but also to identify economical retentate processing solutions in commercial applications. This study also demonstrated that, in addition to pore size, the physico-chemical surface properties of the milk components retained by the membrane can affect the outcome of filtration and thus need to be considered. Overall, there could be additional benefits for microbial removal efficiency by slightly reducing MF membrane pore size to produce low-spore-count milk and dairy

products, which can lead to quality, safety, and economic benefits for the dairy industry.

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