**Microbacterium** represents an emerging microorganism of concern in microfiltered extended shelf-life milk products

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**ABSTRACT**

Growing interest in the manufacture of extended shelf-life (ESL) milk, which is typically achieved by a high-temperature treatment called ultra-pasteurization (UP), is driven by distribution challenges, efforts to reduce food waste, and more. Even though high-temperature, short-time (HTST) pasteurized milk has a substantially shorter shelf life than UP milk, HTST milk is preferred in the United States because consumers tend to perceive UP milk as less desirable due to the “cooked” flavor associated with high-temperature processing. While ESL beyond 21 d may be possible for HTST, the survival and outgrowth of psychrotolerant aerobic spore-forming bacteria can still be a limitation to extending shelf life of HTST milk. Microfiltration (MF) is effective for reducing vegetative microorganisms and spores in raw milk, but it is unclear what the effects of membrane pore size, storage temperature, and milk type (i.e., skim vs. whole) are on the microbial shelf life of milk processed by both MF and HTST pasteurization. To investigate these factors, raw skim milk was MF using different pore sizes (0.8 or 1.2 μm), and then MF skim milk and standardized whole milk (MF skim with heat-treated [85°C for 20 s] cream) were HTST pasteurized at 75°C for 20 s. Subsequently, milk was stored at 3°C, 6.5°C, or 10°C and total bacteria counts were measured for up to 63 d. An ANOVA indicated that mean bacterial concentrations between storage temperatures were significantly different from each other, with mean maximum observed concentrations of 3.67, 5.33, and 8.08 log_{10} cfu/mL for storage temperatures 3°C, 6.5°C, and 10°C, respectively. Additionally, a smaller difference in mean maximum bacterial concentrations throughout shelf life was identified between pore sizes (< 1 log cfu/mL), but no significant difference was attributed to milk type. An unexpected outcome of this study was the identification of *Microbacterium* as a major contributor to the bacterial population in MF ESL milk. *Microbacterium* is a psychrotolerant, thermotolerant, non-spore-forming rod with a small cell size (~0.9 μm length and ~0.3 μm width), which our data suggest was able to permeate the membranes used in this study, survive HTST pasteurization, and then grow at refrigeration temperatures. While spores continue to be a key concern for the manufacture of MF, ESL milk, our study demonstrates the importance of other psychrotolerant, thermotolerant bacteria such as *Microbacterium* to these products. 

**Key words:** fluid milk, spores, *Microbacterium*, extended shelf life, microfiltration

**INTRODUCTION**

The demand for fluid milk products with shelf lives beyond that found in conventional HTST milk (e.g., 14–21 d) is driven by the flexibility that additional shelf life offers to fluid milk supply chains. Ultra-pasteurization (UP) and UHT processing are used to produce extended shelf-life (ESL) and shelf-stable milk, respectively, due to the ability of these processes to destroy bacterial spores that survive the HTST process and subsequently cause spoilage. While UP and UHT milk products provide the benefit of ESL and shelf stability, respectively, HTST milk is the preferred milk type consumed in the US market. This preference is driven by the off-odors and flavors (e.g., cooked) developed during the higher heat treatment of UP and UHT processing (Lee et al., 2017). While UP and UHT are effective at destroying bacterial spores, there are additional technologies such as bactofugation and microfiltration (MF) that are effective at removing spores in raw skim milk before pasteurization, resulting in ESL products.

A limitation of using MF for bacterial removal is that it can only be applied to skim milk for it to be an efficient process (Huppertz et al., 2017). Milkfat globules range in size from 0.2 to 15 μm in diameter, though most milk fat globules are between 1 and 8 μm (Huppertz and Kelly, 2009), and therefore the majority are retained in the retentate during MF (Jukkola et al., 2016). Industrially, when MF is applied for the purpose of microbial reduction, cream is separated and
heat treated at high temperatures before standardization of MF skim (Tomasula and Bonnaillie, 2015). Yet, the impact of standardizing MF skim milk using HTST pasteurized cream on shelf life is not fully understood. Further, there is limited research comparing different MF membrane pore sizes even though it has been shown that bacterial spores are rejected at different rates by membranes with different pore sizes (Griep et al., 2018). Spore-forming bacteria have been considered to be the primary biological limitation to fluid milk shelf life as the small size of spores allows some of them to pass through MF membranes typically used in dairy processing (Griep et al., 2018), and they are also resistant to HTST pasteurization (Huck et al., 2007). Several studies have also identified Microbacterium as a major contributor to the bacterial population in MF milk products. For example, Schmidt et al. (2012) reported that of 250 commercially processed microfiltered ESL milk samples followed through shelf life, nearly 65% of the samples were dominated by Microbacterium at the end of storage, followed by just under 21% of the samples dominated by gram-positive spore-formers (e.g., Bacillus, Paenibacillus, etc.). Microbacterium is a thermotolerant organism, meaning it can survive low to moderate heat treatments in vegetative form (Bulut et al., 1999; Walsh et al., 2012), and at least some strains are able to grow and produce lipolytic and proteolytic enzymes during storage below 10°C (Schmidt et al., 2012). These characteristics, combined with its small cell size, establish the ideal conditions for Microbacterium to emerge as a spoilage organism of concern for the growing microfiltered HTST ESL milk market.

Overall, the primary goal of our study was to evaluate the impact of (1) 2 different membrane pore sizes (i.e., 0.8 and 1.2 μm), (2) standardization of MF skim milk with pasteurized cream, (3) different storage temperatures on the resulting shelf life of MF, standardized fluid milk, and (4) identify bacterial populations driving shelf-life limitation in MF and HTST fluid milk. Ultimately, our study identifies the key microbial drivers of the shelf life of MF and HTST milk, providing important insights into the production of ESL milk manufactured without UP or UHT treatment.

**MATERIALS AND METHODS**

This research involved human participants, but was determined to be exempt for review and oversight by the Cornell University Institutional Review Board.

**Sample Collection, Processing, and Storage**

For each of 4 trials (i.e., biological replicates), raw milk was separated at one of 2 different commercial processing facilities, one in Texas and the other in New York. Approximately 120 L (30 gallons) of raw skim milk and 20 L (6 gallons) of raw cream were collected into 3.8-L (1 gallon) sterile Nalgene bottles (Thermo Fisher Scientific, Waltham, MA) and immediately placed in ice water. Raw skim milk and cream samples continued to be held on ice for up to 24 h including during transportation to Cornell University (Ithaca, NY) as described below, where it was then held at 4°C for up to 24 h before processing. For trial 1, milk was collected from a commercial processing facility in Texas in July of 2021, and samples were shipped in coolers overnight on ice to Cornell. To monitor temperature, a 3.8-L container of raw skim milk containing a temperature data logger was included in each cooler. All product arrived below 6°C. For the remaining 3 trials, raw milk, raw skim milk, and raw cream were collected from a commercial processing facility in New York State between November 2021 and March 2022. Raw milk samples were collected to assess raw milk quality before separation; however, a raw milk sample was not available for the single trial using milk from the Texas processing facility.

All subsequent processing was performed at Cornell University in the Food Processing and Development Laboratory (Ithaca, NY). Approximately 49 L of raw skim milk was commingled into the 50-L holding tank of a pilot-scale MF unit (equipped with a centrifugal pump and tubular heat exchanger), then processed with either a 0.8 or 1.2 μm TAMI ISOFLUX 23 channel tubular, ceramic membrane (TAMI Industries) at 50°C (measured using an in-line temperature probe). The MF process lasted for 1 h, and permeate (MF skim milk) was collected from an in-line valve into a 38-L (10-gallon) milk can. Following the first run (with either the 0.8- or 1.2-μm membrane), the MF unit was fully cleaned and sanitized, and the remaining raw skim milk was MF with the second pore size membrane. Cleaning and sanitation involved (1) a reverse osmosis (RO) water rinse for 10 min, (2) alkaline cleaning by adding 40 L of RO water and 800 mL of Ultrasil-25 (20 g/L) to the holding tank and circulating this solution for 30 min at 80°C, (3) RO water rinse for 10 min, (4) acid cleaning by filling the holding tank with 40 L of RO water and 240 mL of 60% nitric acid solution and circulating for 20 min at 50°C, and (5) RO water rinse for 10 min. Immediately before skim milk was run through the MF unit, a disinfection step was performed by circulating hot water at 80°C to 85°C for 30 min through the unit. The membrane run order alternated between trials (i.e., 0.8-μm membrane was run first for trials 1 and 3, whereas the 1.2-μm membrane was run first for trials 2 and 4). Between runs, membranes were stored in a PVC pipe containing 500 mg/kg peracetic acid.
acid and 2,000 mg/kg hydrogen peroxide solution. Permeate flux was monitored gravimetrically during MF, and water flux was measured after cleaning and sanitation to ensure efficient membrane cleaning.

Raw cream was pasteurized at 85°C for 20 s before the addition to MF skim for standardization. Immediately following MF, skim milk was either pasteurized at 75°C for 20 s or standardized to whole milk (approximately 3.5% fat) with the pasteurized cream. Immediately following standardization, whole milk was also pasteurized at 75°C for 20 s. Pasteurization of the raw cream, MF skim, and standardized whole milk (i.e., MF skim milk standardized with HTST pasteurized cream) was performed using a small-scale HTST pasteurizer (model MicroThermics 25DH) at a flow rate of approximately 1 L/min and then subsequently cooled to 10°C before sample collection. A total volume of 6 L of each milk type (i.e., skim or whole) and each membrane pore size combination (i.e., 0.8 μm skim, 0.8 μm whole, 1.2 μm skim, or 1.2 μm whole) was aseptically collected and then distributed into 250-ml sterile, screw-capped Pyrex bottles (each filled with 200 mL of pasteurized milk). Samples were then stored for shelf-life assessment at 3°C, 6.5°C, or 10°C for up to 63 d or until samples became coagulated. A 500-mL sample of raw skim milk, raw cream, MF skim milk, heat-treated cream, and standardized whole milk (before pasteurization) were all collected for microbiological and physicochemical analyses. For a single trial (trial 1), 0.8-μm data were not collected because the raw milk sample had a bacterial load above 300,000 cfu/mL, which is the U.S. Food and Drug Administration Pasteurized Milk Ordinance (PMO) limit for commingled raw milk. Although the raw skim milk used for 1.2-μm MF processing was from the same processing facility, it was collected the following day and did not exceed the PMO limit; thus, data from 4 trials were available for 1.2-μm MF and 3 trials were available for 0.8-μm MF.

**Microbiological Analyses of Raw Milk and Pasteurized Fluid Milk Samples**

Raw skim milk, raw cream, and MF skim milk were assessed for (1) mesophilic spore count (MSC), (2) psychrotolerant spore count (PSC), and (3) aerobic plate count (APC). Before plating, raw cream was diluted 10-fold (11 g/99 mL) with buffer peptone water that was heated to 40°C to 50°C according to the Standard Methods for the Evaluation of Dairy Products (Duncan et al., 2004) to ensure accurate pipetting. All remaining samples were plated without dilution. The MSC and PSC were performed by heating a 30 mL of each raw milk sample at 80°C for 12 min, followed by cooling on ice and subsequent distribution of 10 mL across 10 standard method agar (SMA) plates (yielding a detection limit of 1 spore per 10 mL or 0.1 spores/mL). Plates were incubated at 32°C for 48 h for MSC or 6°C for 21 d for PSC followed by enumeration. Finally, APC was conducted by spiral plating 50 μL of product onto duplicate SMA plates (2 technical replicates, yielding a detection limit of 1 cfu/50 μL or 20 cfu/mL per plate) using the E 50 mode function (Neutec Eddy Jet 2). Plates were then incubated at 32°C for 48 h before enumeration.

Microbial analysis was conducted on shelf-life samples stored at 3°C, 6.5°C, and 10°C, on d 0, 7, 14, 21, 28, 35, 42, 49, 56, and 63 of shelf life. Microbial shelf-life assessment included (1) enumeration of total APC as described above; (2) a total gram-negative bacteria count, the presence of which would indicate evidence of postpasteurization contamination (PPC), was determined by spiral plating 50 μL of sample onto crystal violet tetrazolium agar (CVTA), followed by incubation at 21°C for 48 h, and enumeration of only red colonies; and (3) a gram-negative stress test on the first day of testing (i.e., d 0) as a rapid indication of whether PPC had occurred. This stress test involved incubating 60 mL of pasteurized milk samples at 21°C for 18 h followed by subsequent plating on CVTA and incubating at 21°C for 48 h before recording the presence or absence of typical growth (i.e., red colonies; Alles et al., 2018). Absence of growth on CVTA was considered a preliminary indication that PPC did not occur during processing; however, we continued to assess all samples for the presence of gram-negative bacteria throughout shelf life as described above, as PPC typically occurs at very low levels and may not be detected during the stress test. In cases where there was evidence of PPC in a sample, all data associated with that sample (i.e., plate counts, pH, particle size, and bacterial identification data) were not included in our analyses. Samples that were clearly coagulated were not plated to prevent clogging of the spiral plater pipetting mechanism; however, some spoiled samples were plated because coagulation was not obvious and the onset of aggregation in those samples was not identified until particle size analysis.

Following incubation, all MSC, PSC, APC, and CVTA plates were enumerated using an automated colony counter (IUL S.A.).

**Physicochemical Analysis**

Both particle size analysis and pH measurements were performed to assess changes in milk across shelf life. Briefly, duplicate samples were prepared separately and then assessed for particle size and pH measurements on each day of shelf life (i.e., d 0, 7, 14, 21, 28, 35,
42, 49, 56, 63). pH was evaluated according to Hooi et al. (2004), using a combination electrode (model Beckman Coulter pH 470). Particle size measurements were taken with a dynamic light scattering particle analyzer equipped with a Peltier temperature control system (Brookhaven Instruments Corp., Holtsville, NY). First, samples were diluted to 0.5% to 1% vol/vol with deionized water inside a 3.5-mL plastic cuvette before taking measurements, to comply with the manufacturer’s recommendation that a signal intensity between 700 and 900 kilo counts per second is obtained. Samples were equilibrated to 25°C within the particle analyzer, and then measurements were taken over a period of 2 min at a fixed 90° angle and a wavelength of 658 nm.

Data collection and analysis was performed using the BIC software (Brookhaven Instruments Corp.), which provided as outputs the particle size distribution, intensity weighted effective diameter, and polydispersity index (p).

Additionally, milk composition analysis was performed using Fourier-transform infrared spectroscopy with a MilkoScan Minor analyzer (FOSS, Denmark) to (1) determine protein loss during MF, and (2) the fat percentage of the standardized milk described above. For each trial, duplicate 60-mL samples of the raw skim milk, MF milk, and standardized milk were equilibrated to room temperature (21°C) before analysis.

**Bacterial Isolation, Preservation, and Subsequent Sequencing and Taxon Assignment**

To assess bacterial populations in raw products and across shelf life, for samples with any growth on MSC, PSC, or APC plated on SMA, colonies with unique morphologies were selected for isolation (typically 1 to 5 isolates). Isolation was performed by sub-streaking colonies onto brain heart infusion (BHI) agar plates and incubating at 32°C for 24 h. Following incubation, if the plate appeared to be a pure culture (i.e., all colonies had the same morphology), a colony was selected for inoculation into 1 mL of BHI broth and incubated at 32°C for up to 48 h. The majority of isolates exhibited growth after 24 h; isolates that exhibited no growth after 24 h were incubated an additional 24 h (a total of 48 h) to allow for growth. Tubes of BHI broth that appeared to be turbid were used to create culture stocks of isolates by mixing 850 μL of the culture with 150 μL of glycerol and stored at −80°C to preserve cultures to allow for further characterization. Following isolation, isolates representing raw products and shelf-life samples were characterized by either rpoB sequencing and allelic type (AT) assignment, as described previously (Gaballa et al., 2021), or 16S rDNA analysis, as described previously (Huck et al., 2007). For AT assignment, rpoB consensus sequences were searched by Basic Local Alignment Search Tool (BLAST) against an internal database (Gaballa et al., 2021), and 16S rDNA consensus sequences were assigned to a genus by using a search against the Ribosomal Database Project Seqmatch database (Center for Microbial Ecology, Michigan State University, East Lansing, MI; Cole et al., 2014). However, for some isolates characterized using 16S rDNA analysis (n = 16), the top 10 matches included more than one genus. For these isolates, the top 10 matches represented the same family, so isolates were assigned to this family.

**Data Curation and Statistical Analysis**

Raw data were organized in Microsoft Excel (Microsoft Excel for Microsoft 365 MSO, version 2207, Build 16.0.15427.20182, 64-bit; Microsoft Corp.) and data wrangling was performed in OpenRefine (version 3.4.1, https://openrefine.org/). Data manipulation, statistical analyses, and creation of plots were all performed using RStudio (version 2022.02.3 build 492 for Windows, RStudio PBC). A 4-way interaction ANOVA was performed in RStudio to assess the effect of (1) pore size (0.8 and 1.2 μm); (2) milk type (skim and whole); (3) storage temperature (3°C, 6.5°C, and 10°C); and (4) day (0, 7, 14, 21, 28, 35, 42, 49, 56, and 63) on bacterial concentration (log_{10} cfu/mL) in milk throughout shelf life. Data manipulation, including log-transformations and grouping and filtering of data for summary statistics, was performed using the rstatix (Kassambara, 2021) and dplyr packages (Wickham et al., 2021). Figures were created with ggplot2 (Wickham, 2016). All raw data files and code used for this paper are available at https://github.com/fsl-nqip/esl_microfiltration.

Samples with MSC, PSC, or APC below the limit of detection were assigned a value of 25% of the detection limit before further data analysis, an approach that has previously been used (Lau et al., 2022). For example, the detection limit per APC plate was 1 cfu/50 μL or 20 cfu/mL. Thus, in this example, samples with no detectable growth were assigned 25% of 20 cfu/mL (5 cfu/mL). Further, if all replicates were below the detection limit, mean log_{10} values are reported as “< x log_{10} cfu/mL” where x is the log_{10} value of the detection limit. Thus, in this example, if all values were below the detection limit of 20 cfu/mL (1.30 log_{10} cfu/mL), the mean would be reported as <1.30 log_{10} cfu/mL.

To estimate the number of days for bacterial concentration to reach important fluid milk microbial concentration thresholds, primary growth models were used to estimate time to the PMO bacterial limit for pasteurized fluid milk (20,000 cfu/mL) and to the concentration where consumers may begin to notice sensory
defects (1,000,000 cfu/mL; Carey et al., 2005). First, the mean APC (log_{10} cfu/mL) of trials was calculated for each combination of day of shelf life, pore size, milk type, and storage temperature (e.g., the mean APC of 0.8-μm MF skim milk stored at samples stored at 6.5°C on d 14). Following this, primary growth parameters were estimated from fitting the model with the lowest Bayesian information criterion (BIC) (model with the lowest Bayesian information criterion). The growth parameters were estimated from fitting the model with the lowest BIC. The growth parameters were estimated from fitting the model with the lowest BIC (BIC; Supplemental Tables S1 and S2, https://github.com/FSL-MQIP/ESL_Microfiltration) used to manually be calculated to fit 3-primary growth models (Buchanan, Baranyi, and modified Gompertz; Zwietering et al., 1990). The initial parameters were calculated for (1) N_0 by taking the mean APC (log_{10} cfu/mL) at d 0 of shelf life; (2) λ by taking the mean time (in hours) between the first 2 consecutive time points where there was a 2-log cfu increase (e.g., if there was a 2-log cfu increase between hours 0 (d 0) and 168 (d 7), 84 h was used as λ); (3) μ_max by taking the greatest log_{10} cfu difference between 2 consecutive time points and dividing by time in hours (e.g., if the greatest log_{10} cfu difference between consecutive time points was 4-log_{10} cfu and was between d 49 and 56 (a span of 168 h), μ_max would be calculated as 4-log_{10} cfu/168 h = 0.024 cfu/h); and (4) N_max by taking the maximum observed APC (log_{10} cfu/mL) across all time points. The growth parameters were estimated from fitting the model with the lowest Bayesian information criterion (BIC; Supplemental Tables S1 and S2, https://github.com/FSL-MQIP/ESL_Microfiltration) and were then used to estimate the time to 20,000 and 1,000,000 cfu/mL using the bigrowth package (Garre et al., 2021). If multiple models had the lowest BIC (e.g., the BIC of Gompertz and Baranyi are equal and both lower than the BIC of Buchanan), the times to 20,000 and 1,000,000 cfu/mL were calculated for each model with the lowest score and then the mean time was reported.

Nonmetric multidimensional scaling (NMDS) analysis was performed to determine if differences were present in the populations of milk processed with different pore size membranes, different milk types, storage temperatures, or trials. Analysis of similarities (ANOSIM), an ANOVA-like test, was used to determine if there was a difference in genera, species, or rpoB AT between (1) MF membrane pore size, (2) milk type, (3) storage temperature, and (4) trials. If a significant difference was found with the ANOSIM test, multilevel pattern analysis was performed for determining which genera, species, or AT, if any, is significantly associated with one or more pore sizes, milk type, storage temperature, or trial. Before and after removing singletons (i.e., an isolate representing a single genus, species, or rpoB AT that is only represented once across all samples or an isolate that is the only genus, species, or rpoB AT present for a unique combination of pore size, milk type, storage temperature, and trial), we were not able to reach convergence for NMDS analysis at the AT or genera level, as data were not sufficient (i.e., not enough distinct samples to allow for NMDS).

**RESULTS**

**Bacterial Counts and Component Analysis of Raw Materials, Microfiltered Skim, Heat-Treated Cream, and HTST Finished Product**

The APC for all raw milk (n = 3), raw skim (n = 4), and raw cream (n = 4) had combined trial mean concentrations of 4.58 (range 4.12–5.13) log_{10} cfu/mL, 4.12 (range 3.04–5.35) log_{10} cfu/mL, and 3.15 (range 1.58–4.50) log_{10} cfu/g, respectively. The mean APC for raw skim milk used for 0.8-μm MF runs and for 1.2-μm MF runs was 3.83 (range 3.05–5.35) and 4.12 (range 3.00–5.36) log_{10} cfu/mL, respectively, whereas the mean APC of MF skim was 2.01 (range 1.00–3.07) and <1.30 log_{10} cfu/mL (all APC below the detection limit) for 0.8 and 1.2 μm runs, respectively (Table 1). Thus, a mean reduction of 1.82 and >2.82 log_{10} cfu was achieved by MF with 0.8- or 1.2-μm membranes, respectively (Table 1). Subsequent pasteurization of MF skim milk resulted in a mean APC of 1.77 and 0.78 log_{10} cfu/mL for milk from pore size runs with 0.8 and 1.2 μm, respectively, resulting in an overall mean reduction of 2.06 and 3.34 log_{10} cfu from the raw milk to the finished product, respectively.

The mean APC for cream after HTST pasteurization at 85°C for 20 s was 1.14 (range <1.30–1.65) log_{10} cfu/g. Additionally, the mean APC for standardized whole milk before pasteurization (i.e., MF raw skim with added pasteurized cream) was 2.06 and 1.21 log_{10} cfu/mL with mean APC immediately after HTST pasteurization (i.e., d 0 of shelf life) of 1.69 and 1.15 log_{10} cfu/mL for 0.8 and 1.2 μm runs, respectively. Pasteurization of whole milk resulted in a mean reduction of 0.37 and 0.06 log_{10} cfu/mL from standardized MF milk to HTST finished product for 0.8 and 1.2 μm runs, respectively.

The MSC and PSC were performed for raw skim, raw cream, and MF skim, and for all trials combined, the overall MSC means were 1.01, 0.73, and −0.25 log_{10} cfu/mL and overall PSC means were −0.37, −0.11, and <−1.00 log_{10} cfu/mL (all MF skim milk PSC were below the detectable limit of −1.0 log_{10} cfu/mL), respectively. The mean MSC for raw skim used for 0.8- and 1.2-μm MF runs was 1.03 and 0.88 log_{10} cfu/mL, respectively (Table 1). The mean PSC for raw skim used for 0.8- and 1.2-μm MF runs was −0.50 and −0.22 log_{10} cfu/mL, respectively (Table 1). The mean MSC for 0.8- and 1.2-μm MF skim was −0.35 and 0.00 log_{10} cfu/mL, respectively, whereas all PSC for both
0.8- and 1.2-μm MF skim were below the detection limit of –1.00 log₁₀ cfu/mL (Table 1). Thus, MF of raw milk with 0.8- and 1.2-μm membranes resulted in a mean reduction (calculated as the mean raw skim count minus the mean MF skim count) of 1.38 and 0.88 log₁₀ cfu/mL for mesophilic spores, respectively, and >0.50 and >0.78 log₁₀ cfu/mL for psychrotolerant spores, respectively.

Additionally, infrared composition analysis was performed on raw and MF skim milk to determine the percent protein retention by each membrane. For both 0.8- and 1.2-μm membranes, the mean protein retention was 1.7%. For the 0.8-μm membrane, the percent protein retention was 1.8, 3.7, and 0.0 for trials 2, 3, and 4, respectively. For the 1.2-μm membrane, the percent protein retention was 2.6, 2.8, 2.0, and 0.0 for trials 1, 2, 3, and 4, respectively (Supplemental Table S3, https://github.com/FSL-MQIP/ESL_Microfiltration).

**Effect of Pore Size, Storage Temperature, and Milk Type on Aerobic Plate Counts of Fluid Milk**

We performed APC to quantify bacterial concentration across 63 d of shelf life of microfiltered milk stored at different refrigeration temperatures. For storage temperatures 3°C (Figure 1a), 6.5°C (Figure 1b), and 10°C (Figure 1c), the highest mean bacterial concentrations of milk (calculated as the mean of the highest concentration for each trial) ± 1 standard deviation from the mean were 3.35 ± 1.53, 5.02 ± 2.08, and 7.83 ± 0.82 log₁₀ cfu/mL, respectively. The highest mean concentrations and standard deviations were 5.84 ± 2.18 and 5.07 ± 2.56 log₁₀ cfu/mL for milk microfiltered with pore sizes 0.8 and 1.2 μm, respectively. The highest mean concentrations of skim and whole milks were 5.14 ± 2.49 and 5.66 ± 2.36 log₁₀ cfu/mL, respectively.

Based on the ANOVA performed to assess the effect of pore size, milk type, storage temperature, and day, we observed substantial differences between APC between storage temperature, day, and the interaction of storage temperature and day (all P < 0.001). For the partial η² measure of effect size calculated here, ≤0.01, 0.06 to 0.13, and >0.14 indicate small, medium, and large effects, respectively (Cohen, 1988). The effect sizes for storage temperature and day were large (η² = 0.22 and η² = 0.32, respectively). A difference in APC was also observed between pore sizes, and there was a large effect of size (P < 0.05, η² = 0.16), but no difference was observed between milk types (P = 0.33). The ANOVA for assessing the effect of the predictor variables on bacterial concentration was subsequently followed by Tukey’s tests for storage temperature and pore size. We found with these tests that the bacterial concentrations at 3°C and 6.5°C were significantly different from each other (P < 0.01) and were both significantly different from 10°C (both P < 0.0001); however, there was no significant difference in bacterial concentration between membrane pore sizes (P = 0.12).

Additionally, the time to reach important milk quality thresholds was estimated (Table 2) as described in the Data Curation and Statistical Analysis section. Because we lacked data ≥20,000 cfu/mL for all milk stored at 3°C and milk MF with a 1.2-μm membrane and stored at 6.5°C, and also lacked data ≥1,000,000 cfu/mL for both 3°C and 6.5°C, estimates could not be made at these thresholds for these storage temperatures. Further, the observed trial mean Ymax of 6.37 log₁₀ cfu/mL was used instead of the value 5.50 log₁₀ cfu/mL obtained from fitting the data to the modified Gompertz model because using 5.50 log₁₀ cfu/mL did not allow us to predict time to 1,000,000 cfu/mL (Supplemental Table S1). Finally, for datasets that

### Table 1. Aerobic plate counts, mesophilic spore counts, and psychrotolerant spore counts of raw skim milk before and after microfiltration with 0.8- or 1.2-μm membranes

<table>
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<th>Trial</th>
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<th>Microfiltered skim</th>
<th>Reduction¹</th>
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<td>&lt;1.00</td>
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<td>&lt;1.00</td>
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<td>2.05</td>
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<td>&lt;0.51</td>
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<td>&lt;1.00</td>
<td>&lt;1.00</td>
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<td>&lt;1.30</td>
<td>&gt;1.78</td>
<td>0.56</td>
<td>&lt;1.00</td>
<td>&gt;1.56</td>
<td>0.52</td>
<td>&lt;1.00</td>
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<tr>
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<td>3.09</td>
<td>1.95</td>
<td>1.14</td>
<td>0.20</td>
<td>1.10</td>
<td>&gt;0.90³</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
<td>&gt;0.64</td>
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<td>4</td>
<td>1.2</td>
<td>3.00</td>
<td>&lt;1.30</td>
<td>&gt;1.70</td>
<td>0.40</td>
<td>&lt;1.00</td>
<td>&gt;1.40</td>
<td>&lt;1.00</td>
<td>&lt;1.00</td>
<td>&gt;0.70</td>
</tr>
</tbody>
</table>

¹Reduction is reported as the raw skim plate count minus the microfiltered skim count.

²Not available. Some reduction values could not be computed (i.e., if the raw skim milk count and the microfiltered skim test were below the detectable limit).

³The only case where the microfiltered skim bacterial count was greater than the corresponding raw skim bacterial count. Sample or media contamination cannot be ruled out as a cause of this data point.
Figure 1. Aerobic plate counts (log10 cfu/mL) of (1) skim milk (microfiltered with a 0.8- or 1.2-μm filtration membrane followed by pasteurization at 75°C for 20 s) or (2) whole milk (skim milk microfiltered with a 0.8- or 1.2-μm filtration membrane followed by the addition of heat-treated cream (85°C for 20 s) and pasteurized at 75°C for 20 s); then (1) skim milk and (2) whole milk were stored at (a) 3°C, (b) 6.5°C, or (c) 10°C for up to 63 d. The function geom_smooth from ggplot2 (Wickham, 2016) was used to fit the points with a regression line using the locally estimated scatterplot smoothing (LOESS) method.
did not surpass a certain threshold (i.e., 20,000 cfu/mL or 1,000,000 cfu/mL), the time to that threshold was assumed to be greater than 63 d (the last day of tested shelf life). For example, the mean datasets for all samples stored at 6.5°C did not surpass 1,000,000 cfu/mL, and we therefore assume the time to this 1,000,000 cfu/mL to be >63 d (Table 2).

The predicted time to 20,000 and 1,000,000 cfu/mL was much shorter for milk stored at 10°C compared with milk stored at 6.5°C (Table 2). For example, for whole milk obtained using skim milk MF with a 1.2-μm membrane, the predicted time to 20,000 cfu/mL was >63 and 11 d for milk stored at 6.5°C and 10°C, respectively. Also, predicted time to thresholds was shorter for milk MF with a 0.8-μm membrane compared with milk MF with a 1.2-μm membrane. For example, the predicted time to 20,000 cfu/mL was 37 and >63 d for skim milk stored at 6.5°C and microfiltered with 0.8 and 1.2 μm, respectively. When comparing milk types, the difference in predicted time to 20,000 and 1,000,000 cfu/mL between skim and whole milk was considerably smaller. For milk MF with a 0.8-μm membrane and stored at 6.5°C, the predicted time to 20,000 cfu/mL for skim and whole milk was 37 and 42 d, respectively.

**Characterization of Bacteria Isolated from Different Milk Types That Were Subjected to Microfiltration with Different Pore Sizes and Storage Temperatures**

A total of 288 isolates were collected from APC of shelf-life samples and raw milk tests. Of these isolates, 278 were successfully characterized by rpoB or 16S rDNA PCR and subsequent sequencing, with 125/278 and 153/278 isolates characterized by rpoB and 16S, respectively. To select the isolates that were representative of bacteria that survived processing and grew throughout shelf-life samples, isolates from the 3 shelf-life days with the highest APC for unique combination of trial-membrane pore-size-milk type-storage temperature (e.g., trial 2, 0.8, whole, 6.5°C) were selected for characterization. From this process, 110 isolates were selected from the initial set of 278, with 43 and 67 isolates being characterized by rpoB and 16S, respectively. After removing duplicate isolates with the same rpoB AT, that is, isolates that had the same AT for rpoB and came from a single sample with a unique combination of trial, milk type, membrane pore size, storage temperature, and day of shelf life (e.g., for trial 1 of 0.8-μm MF skim milk stored at 6.5°C on d 49 of shelf life), we were left with 102 isolates representing unique family or genera (n = 67, characterized by 16S) or species and rpoB AT (n = 35, characterized by rpoB) originating from a given trial, pore size, milk type, storage temperature, and day of shelf life. Out of this subset, 10 isolates were characterized as gram-negative organisms at the family (n = 5) or genus (n = 5) level and removed from further analysis. These 10 isolates were unique to 10 different samples and these samples were removed from statistical analyses for plate count, pH, and particle size data. Overall, for the remaining 92 isolates, there were 5 unique genera, 9 unique species, and 20 unique rpoB AT. Overall, the top genus, species, and rpoB allelic type isolated were Microbacterium (n = 51), Bacillus mosaicus (n = 12), and Bacillus mosaicus AT 61 (n = 7), respectively. For skim milk isolates (n = 36), the top genus, species, and rpoB allelic type were Microbacterium (n = 25), Bacillus mosaicus (n = 5), and Bacillus mosaicus AT 410 (n = 4), respectively, whereas for whole milk isolates (n = 56), the top genus, species, and rpoB allelic type were Microbacterium (n = 26), Bacillus mosaicus and Paenibacillus odorifer (both, n = 7), and Bacillus mosaicus AT 61 (n = 6), respectively (Figure 2c). Additionally, when comparing isolates collected from milk

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**Table 2.** Average predicted time (d) to 20,000 and 1,000,000 cfu/mL for microfiltered (0.8- or 1.2-μm membrane) skim and whole milk pasteurized at 75°C for 20 s and stored at 6.5 or 10°C.1,2

<table>
<thead>
<tr>
<th>Item</th>
<th>6.5°C</th>
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<th>10°C</th>
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<tbody>
<tr>
<td></td>
<td>Skim</td>
<td>Whole</td>
<td>Skim</td>
<td>Whole</td>
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<tr>
<td>Days to 20,000 cfu/mL, range (mean)</td>
<td>0.8</td>
<td>1.2</td>
<td>0.8</td>
<td>1.2</td>
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<td></td>
<td>37</td>
<td>&gt;63</td>
<td>42</td>
<td>&gt;63</td>
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<tr>
<td>Days to 1,000,000 cfu/mL, range (mean)</td>
<td>&gt;63</td>
<td>&gt;63</td>
<td>&gt;63</td>
<td>&gt;63</td>
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</table>

1Time to 20,000 and 1,000,000 cfu/mL could not be calculated for all milk stored at 6.5°C because there were not enough data near 1,000,000 cfu/mL; however, based on the shelf-life data for 6.5°C, it is reasonable to assume that time to 1,000,000 cfu/mL is more than 63 d. Similarly, for both 1.2-μm filtered skim and whole milk stored at 6.5°C, mean time to 20,000 cfu/mL could not be calculated, and thus is assumed to be more than 63 d.

2Predicted times are based on first fitting aerobic plate count data and estimated primary growth parameters to 3 primary growth models (i.e., Buchanan, Baranyi, and modified Gompertz). The model with the lowest Bayesian information criterion was selected for calculating the time to thresholds.
MF with different membrane pore sizes, the top genus, species, and rpoB allelic type was Microbacterium (n = 27), Bacillus mosaicus (n = 9), and Bacillus mosaicus AT 61 (n = 7), for milk microfiltered with a 0.8-μm membrane (n = 54); and Microbacterium (n = 24), Bacillus mosaicus (n = 3), and Bacillus mosaicus AT 410 (n = 3), for milk MF with a 1.2-μm membrane (n = 38; Figure 2b). Last, comparing the different storage temperatures used in this study, the top genus, species, and rpoB allelic type (1) for isolates collected from milk held at 3°C (n = 9) were Bacillus (n = 3), Bacillus safensis (n = 3), and Bacillus safensis AT 140 (n = 3), respectively; (2) for isolates collected from milk held at 6.5°C (n = 35) were Microbacterium (n = 24), Bacillus mosaicus and Paenibacillus odorifer (for both, n = 3), and Bacillus mosaicus AT 61 (n = 3), respectively; and (3) for isolates collected from milk held at 10°C (n = 48) were Microbacterium (n = 25), Bacillus mosaicus (n = 12), and Bacillus mosaicus AT 410 (n = 5), respectively (Figure 2d). It should be noted that the 16S rDNA sequencing method employed in this study is not sufficiently discriminatory to confidently assign species identification to Microbacterium. Further, the subtyping gene rpoB could not be used for the Microbacterium isolates, and no appropriate similar subtyping gene is available for this genus. These limitations could have affected our species and subtype (i.e., rpoB) level results in this study, as we only have taxonomic designations for aerobic spore-forming bacteria.

The NMDS analysis and subsequent ANOSIM tests were performed on the 80 isolates described above to determine if differences were present in the populations of milk processed with different pore size membranes, different milk types, storage temperatures, or trials. At the level of species, it was found that storage temperature (P < 0.05) and trial (P < 0.001) were significant, whereas membrane pore size (P = 0.85) and milk type (P = 0.28) were not significant. Following ANOSIM, we performed a multilevel pattern analysis for determining what species, if any, was significantly associated with the level of species, it was found that storage temperature, and milk type were significantly associated with 6.5°C or 10°C (P < 0.05), whereas no trial was significantly associated with storage temperature of 3°C (P = 0.1). Further, Microbacterium was also significantly associated with 3°C and 10°C (P < 0.05), whereas no species were significantly associated with 6.5°C or 10°C storage temperature.

To assess what organisms may have initially been present in raw milk and were present in the shelf-life samples where exponential growth was observed, isolates were characterized from APC of milk products before pasteurization (e.g., MF skim milk, raw cream). These isolates represented 39 of the 278 isolates characterized, and after removing duplicates, this represented 33 isolates. Duplicates were removed to prevent over-representation of genera and were considered to be isolates with the same genus from the same milk type and trial (e.g., raw cream used for trial 3) or (2) isolates with the same genus from the same milk type, trial, and membrane pore size (e.g., 0.8-μm MF skim from trial 4). These 33 isolates were from raw milk (n = 5), raw cream (n = 4), raw skim (n = 16), MF skim (n = 3), and standardized whole milk before pasteurization (n = 5; Figure 3). The top genus among all 33 isolates was Microbacterium, and Microbacterium was the genus most frequently isolated from raw skim (n = 7) and MF skim (n = 2); however, Microbacterium was also found in raw milk (n = 1), raw cream (n = 1), and standardized milk (n = 2). Streptococcus represented the second most frequently isolated genus (n = 8) among the 33 isolates. Raw milk product isolates were not collected from trial 1 but were collected from the remaining 3 trials.

As Microbacterium was found in all raw milk types before MF (Figure 3), and also represented most isolates collected from shelf life (Figure 2), there is evidence that, in this study, Microbacterium was already present in the raw milk, permeated through the 2 MF membranes, survived pasteurization, and was able to grow over shelf life. Bacillus was also found in the raw milk and in shelf-life samples; however, Bacillus as a genus was not found in raw cream or MF skim, and Bacillus represented a smaller number of isolates compared with Microbacterium across shelf life. Other than Microbacterium, Staphylococcus and Rhodococcus were also non-spore-forming gram-positive organisms found in shelf-life samples, but represented a small number of isolates. Further, Staphylococcus and Rhodococcus were not found in any milk (e.g., raw skim, MF skim) before HTST pasteurization.

Effect of Pore Size, Storage Temperature, and Milk Type on Physicochemical Spoilage-Associated Parameters (pH and Particle Size)

In addition to microbiological analysis, we further characterized milk samples by measuring physicochemical parameters including pH and particle size (Supplemental Figure S1, https://github.com/FSL-MQIP/ESL_Microfiltration). Overall, the mean pH for all samples on d 0 was 6.67 (range 6.49–6.75), whereas the pH on d 63 was 6.66 (range 6.60–6.72), 6.44 (range 5.73–6.72), and 6.25 (range 5.68–6.70) for samples stored at 3°C, 6.5°C, and 10°C, respectively. The pH for milk stored at 3°C, 6.5°C, and 10°C, ranged between 6.49–6.92, 5.73–6.88, and 5.41–6.91, respectively. Low pH values (i.e.,
Figure 2. Characterization of bacteria (n = 92) isolated from skim milk microfiltered with a 0.8- or 1.2-μm filtration membrane followed by (1) pasteurization at 75°C for 20 s or (2) addition of heat-treated cream (85°C for 20 s) and pasteurized at 75°C for 20 s; then (1) skim milk and (2) whole milk were stored at 3°C, 6.5°C, or 10°C for up to 63 d. Species are compared across (a) trials, (b) pore sizes (μm), (c) milk types, and (d) storage temperatures (°C).
below 6.5) were observed more frequently with increased storage time and at higher storage temperatures (i.e., 10°C). Higher than normal pH values (i.e., above 6.8) were observed in some samples throughout shelf life, which may be explained by water inadvertently added during processing. This is supported by measurements of milk freezing point taken immediately after pasteurization for trials 3 and 4 (Supplemental Table S4, https://github.com/FSL-MQP/ESL_Microfiltration).

Particle size analysis showed that the mean effective diameter for milk MF with a 0.8- or 1.2-μm membrane ranged between 161–627 nm and 128–603 nm, respectively, across shelf life. For skim (Supplemental Figure S1c) and whole milk (Supplemental Figure S1d), the mean effective diameter across all days of shelf life (d 0–63) ranged between 128–222 and 227–627 nm, respectively (Supplemental Figure S1d). The mean effective diameter for milk stored at 3°C, 6.5°C, or 10°C, across all days of shelf life, ranged between 158–602 nm, 158–510 nm, and 128–627 nm, respectively. Further, data generally supported evidence of aggregation with increased storage time. For skim milk, the mean particle sizes on d 0 for all storage temperatures were 175 and 171 nm for milk MF with a 0.8- or 1.2-μm membrane, respectively, whereas mean particle sizes on d 63 were 182 and 181 nm, respectively. For whole milk, the mean particle sizes on d 0 for all storage temperatures were 270 and 280 nm for milk MF with a 0.8- or 1.2-μm membrane, respectively, whereas mean particle sizes on d 63 increased to 405 and 383 nm, respectively. Throughout shelf life, greater aggregation occurred in milk samples with higher fat content, as demonstrated by the particle size of whole milk samples increasing faster across shelf life than the particle size of skim milk samples. Additionally, 35 samples were classified as visually coagulated by a single observer. All 35 of these samples were stored at 10°C for at least 14 d. Of these 35 samples, 13 were MF with the 1.2-μm membrane, whereas 22 were MF with the 0.8-μm membrane, and 17 were skim and 18 were whole milk. Particle size measurements for the coagulated samples were not considered accurate, as values were outside of the accuracy range for the instrument, and thus were not included in the descriptive statistics summarized previously.

**DISCUSSION**

Achieving ESL using solely HTST pasteurization of milk is generally not feasible due to the presence of psychrotolerant spore-formers in the raw milk supply that can survive pasteurization, grow under refrigerated storage conditions, and cause quality defects. Microfiltration provides the opportunity to remove a considerable proportion of these spores from raw skim milk before pasteurization. Here we assessed the shelf life of HTST milk subjected to (1) MF with 2 different pore size membranes (0.8 and 1.2 μm) before HTST pasteurization, (2) standardization of MF skim milk using...
HTST pasteurized cream before HTST pasteurization, and (3) storage at different refrigeration temperatures for up to 63 d. Our results indicate that MF of skim milk before HTST pasteurization or standardization to whole milk followed by HTST pasteurization resulted in longer estimated time to reach important microbial thresholds (i.e., 20,000 or 1,000,000 cfu/mL) than for fluid milk without MF, especially at lower storage temperatures (i.e., 3°C and 6.5°C). We also observed that milk MF with a 1.2-μm membrane showed lower microbial growth over shelf life compared with milk MF with a 0.8-μm membrane. Finally, while much attention has been paid to the impact of spore-forming bacteria on the shelf life of fluid milk, our results clearly show the role of other thermotolerant organisms, specifically Microbacterium, which represents a biological barrier to the shelf life of MF, HTST, ESL milk.

**Microbacterium Represents a Non-Spore-Forming Bacteria of Concern for Extended Shelf-Life Microfiltered Fluid Milk**

The use of MF to remove bacterial contaminants often focuses on reduction of spore levels in raw milk due to the ability of these organisms to survive HTST pasteurization and subsequently grow to spoilage levels at low temperatures during refrigerated storage (Huck et al., 2007; Ivy et al., 2012; Masiello et al., 2014; Doll et al., 2017). However, in the study presented here we observed that in 3 of the 4 trials, where raw milk was collected from a NY commercial processing facility, Microbacterium, a gram-positive, non-spore-forming rod, was the primary driver of bacterial growth in MF skim and whole milk over shelf life, representing 51 of 89 isolates collected from trials 2 to 4. Microbacterium was also isolated in this study from raw skim milk, representing 7 of 16 isolates collected from raw skim milk APC plates, from trials 2 to 4, indicating that this organism was present before MF. Some strains of Microbacterium have been shown to exhibit a high degree of heat resistance. A recent study describing a new species isolated from microfiltered milk, Microbacterium paulum, indicated that after heat treatment at 75°C/15 s, there was only a 0.1 ± 0.1 log cfu/mL reduction in the cell count of M. paulum, whereas heat treatment at 85°C/15 s resulted in a reduction of 3.4 ± 0.1 log cfu/mL (Bellassi et al., 2021). These findings are consistent with other studies that have identified Microbacterium in HTST pasteurized milk (Kikuchi et al., 1996; Fromm and Boor, 2004), indicating that this organism may survive heat treatment under conventional pasteurization conditions. Importantly, Microbacterium is also capable of producing biofilms (Bellassi et al., 2021) and has historically been associated with poorly cleaned milking equipment (Washam et al., 1977), establishing potential farm and pre-pasteurization sources and contamination pathways throughout the fluid milk continuum.

Our study is not the first to identify Microbacterium in MF milk. For example, Schmidt et al. (2012) evaluated the bacterial populations in 250 MF and HTST pasteurized milks commercially processed by 5 different manufacturers in Germany, Austria, and Switzerland. The authors report that Microbacterium dominated the populations in these samples, being isolated from nearly 65% of the commercial products. Similarly, Bellassi et al. (2021) reported that Microbacterium was the predominant bacterial contaminant in samples of Italian MF milk. Similarly to spores, the small cell size of Microbacterium allows it to pass through commonly used MF membrane pore sizes. Bellassi et al. (2021) reported that 2 Microbacterium isolates collected from MF milk had mean cell lengths of 1.34 ± 0.5 μm and 0.89 ± 0.5 μm, and mean cell diameters of 0.41 ± 0.4 μm and 0.32 ± 0.3 μm, making them small enough to pass through MF membranes typically used for microbial removal in the dairy industry. Further, consistent with our results, various reports indicate that Microbacterium is capable of growing at refrigerated temperatures (Washam et al., 1977; Fromm and Boor, 2004; Schmidt et al., 2012; Bellassi et al., 2021). Additionally, some strains produce a variety of enzymes that may degrade milk protein and lipids during shelf life, ultimately leading to product spoilage (Hantsis-Zacharov and Halpern, 2007; Bellassi et al., 2021).

The ability of Microbacterium to form biofilms, combined with their small cell size, robust heat resistance, ability to grow at low temperatures, and ability to produce proteolytic and lipolytic enzymes make this organism a primary concern for manufacturers of MF ESL fluid milk in addition to aerobic spore-forming bacteria. In particular, our results and previous literature indicate that manufacturers should monitor raw milk for the presence of Microbacterium at both the farm as well as at pre-processing steps (e.g., cream separation) in the manufacturing facility. Future research should focus on identifying high-risk sources for Microbacterium at the farm and in the processing facility, as well as development of microbiological methods that specifically target this organism of concern.

**Addition of Heat-Treated Cream to Microfiltered Skim Milk Does Not Result in a Reduction of Shelf Life**

Our results confirm previous reports that MF is a viable processing tool to substantially extend the shelf life of fluid milk by removing bacterial contaminants from raw milk (Trouvé et al., 1991; Pafylias et al., 1996; Elwell and Barbano, 2006; Fritsch and Moraru,
2008), but we also show that the addition of HTST pasteurized cream to MF skim for standardization of fat content does not result in a reduction of shelf life. We found that the predicted time to reach 20,000 cfu/mL for skim MF with 0.8- and 1.2-μm membranes, then standardized to whole milk, was 37 and 42 d when stored at 6.5°C, respectively. The effect of cream used for standardization of MF skim milk was previously studied by Caplan and Barbano (2013) who reported that 2% MF and HTST pasteurized milk achieved a 60- to 90-d shelf life under refrigeration (i.e., 5.7°C) when raw cream with an extremely low bacteria count (i.e., 25 cfu/mL total bacteria count) was used for standardization before HTST pasteurization of the standardized product. The mean APC of raw cream used in the study described here was substantially higher than that reported by Caplan and Barbano (2013) at 3.15 log₁₀ cfu/mL (~1,400 cfu/mL). Despite the higher total bacteria count of raw cream used in this study, pasteurization of the cream before standardization reduced the total bacteria count to 1.15 log₁₀ cfu/g (~14 cfu/g), which is similar to the bacterial load of the raw cream used by Caplan and Barbano (2013). Our results indicate that raw cream, even with higher initial bacterial loads, can be used to manufacture ESL fluid milk products when it is pasteurized before standardization of MF skim milk. This outcome is of importance to the dairy industry as raw milk used in the manufacture of ESL products may not always be of exceptional quality or may vary from load to load within a manufacturing facility. It should be noted, however, that while microbial quality of fluid milk over shelf life in our study was not affected by the relatively high total bacteria counts (i.e., mean APC of 4.58 log₁₀ cfu/mL) in the raw milk used for processing, using poor quality raw milk for ESL fluid milk is not advisable as native milk and bacterial enzymes produced before processing are likely to result in quality degradation over long storage periods. This nonmicrobial quality degradation was observed by Elwell and Barbano (2006), where MF skim milk held at 6.1°C for 92 d, showed sufficient proteolytic activity to limit the shelf life by 32 d of storage, despite having total bacteria counts below 1,000 cfu/mL, suggesting the need for future sensory studies focused on nonmicrobial spoilage for MF, HTST, ESL milks.

**Pore Size Had a Noticeable Impact on the Shelf Life of Microfiltered Milk, Which Could Be Attributed to Membrane Fouling**

While our findings show that MF combined with HTST pasteurization can result in ESL milk, in this study MF with the 0.8-μm membrane resulted in shorter shelf life compared with MF with the 1.2-μm membrane. This result is counterintuitive and contrary to prior reports. For example, the use of a smaller pore size (i.e., 0.5 μm) was reported by Lindquist (1998) to result in 2 to 3 log larger reduction in bacterial load in skim milk compared with a 1.4-μm membrane (Fernández García et al., 2013). Additionally, Griep et al. (2018) found a greater reduction of Bacillus licheniformis spores when milk was MF with a 1.2-μm membrane compared with a 1.4-μm membrane.

We considered the possibility of bacterial contamination of the 0.8-μm membrane. However, our bacterial characterization data show no evidence of bacterial genera, species, or rpoB AT that represented a significant portion of isolates collected from the finished product of milk MF with a 0.8-μm membrane and was not already present in the raw milk. Further, the ANOSIM analysis of bacterial species present in milk over shelf life showed no significant difference in species populations between pore sizes, providing further evidence that no microbial contamination originated from the 0.8-μm membrane. The most likely explanation for these results, which were consistent across all trials, is that the different fouling status of the 2 membranes. Permeate flux was higher during processing with the 1.2-μm membrane as compared with the 0.8-μm membrane (Supplemental Figure S2, https://github.com/FSL-MQIP/ESL_Microfiltration), which is in line with what was expected based on membrane pore size (Lindquist, 1998). However, the 1.2-μm membrane used in the trials had been used before and had a much lower water flux (~700 L/m²h) than the (virtually new) 0.8-μm membrane (water flux ~1,020 L/m²h), which suggests some irreversible fouling from prior use of the 1.2-μm membrane. This has implications on the membrane surface properties, which can affect the interaction between the membrane and microbial cells. Tan et al. (2014) demonstrated that repeated use of ceramic MF membranes, similar to the ones used in the current study, resulted in irreversible fouling due to protein adsorption, which affected the electrostatic charge (measured by zeta potential) of the membrane. Specifically, the membrane surface became more negatively charged, which could make it more repellant toward microbial cells, which are expected to also be negatively charged. While this hypothesis was not tested in the current study, it is a plausible explanation, and it is consistent with previous reports that membrane fouling increases bacteria retention (Kaufmann and Kulozik, 2006; Cogan and Chellam, 2008).

A practical implication of this observation for the current study and for the industrial use of MF for microbial removal from skim milk is that it may not be necessary to use pore sizes as small as 0.8 μm, because depending on processing conditions and membrane...
surface properties it is possible to effectively remove microorganisms with a 1.2-μm membrane. This would present practical advantages because of higher fluxes and consequently faster processing of milk.

Refrigerated Microfiltered/HTST Fluid Milk Has Substantially Longer Shelf Life Compared with Previously Reported Refrigerated HTST Fluid Milk Shelf Life

Results from our study confirm that refrigerated storage temperature significantly affects the microbial shelf life of MF and HTST pasteurized fluid milk, with product held under temperature abuse conditions (i.e., 10°C) reaching important quality thresholds considerably faster than the product held at 3°C or 6.5°C. Previous studies have also reported bacterial counts over shelf life of microfiltered milk under various storage conditions. Wang et al. (2019) reported that fluid milk cold MF with a 1.4-μm membrane did not exceed a total bacteria count of 20,000 cfu/mL over 92 d when stored at 6°C, supporting our finding that the predicted time to 20,000 cfu/mL was >63 d for milk MF with a 1.2-μm membrane. Similarly, Schmidt et al. (2012) investigated microbial diversity and shelf life among retail MF milk products from Germany, Austria, and Switzerland. In their study, total bacteria counts of whole MF (1.4-μm membrane) milk stored at 4°C did not exceed 20,000 cfu/mL in 2 of 3 trials through 29 d of storage, whereas in MF milk stored at 8°C and 10°C, counts exceeded 5-log10 cfu/mL for all 3 trials by 29 d of storage. Comparably, in the current study, 1 of 14 samples stored at 3°C surpassed 20,000 cfu/mL by d 28 of storage, whereas 12 of 14 samples stored at 10°C surpassed 5-log10 cfu/mL at the same point in shelf life. Our results and those of similar studies indicate that the importance of storage temperature cannot be understated. These results will be important for processors who want to maintain the ESL of MF fluid milk through various distribution channels, including through E-commerce or exports where stringent temperature control may not be feasible.

CONCLUSIONS

This study demonstrates that manufacturing ESL HTST milk is achievable by MF of raw skim milk before HTST pasteurization, even with the addition of heat-treated cream for standardization. Our study further highlights the emergence of non-spore-forming thermoduric bacteria, specifically Microbacterium, as a contaminant of concern for MF milk products due to its small cell size, heat resistance, and ability to grow at low temperatures. Future studies should focus on identifying on farm and pre-pasteurization harborage sites for Microbacterium and further characterizing strain-to-strain variability for parameters that pose a risk to ESL fluid milk products (e.g., cell size, heat resistance). Importantly, we conclude that controlling for Microbacterium in the raw milk supply and the processing environment will be essential for producing high-quality microfiltered HTST milk with extended shelf life.

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