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# Influence of feed temperature to biofouling of ultrafiltration membrane during skim milk processing

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ABSTRACT

The temperature of the feed is known to be an important factor affecting the formation and the growth rates of bacterial communities on dairy filtration membranes. However, decades after the integration of filtration processes in the dairy industry, there is still questioning whether filtration should be performed at cold (<15 °C) or hot (>48 °C) temperature. A biofilm reactor designed to mimic a filtration system was used to provide answers to this question. Bacteria adhering and growing on ultrafiltration membranes in contact with pasteurised skim milk were characterised at 15 °C and 50 °C through a metabarcoding approach. Our results suggested that the processing time should be limited to 10 h at 50 °C to avoid the exponential growth of thermophilic spore-former bacteria, while the use of 15 °C combined with daily cleaning procedures appeared the best way to retard the formation of biofilms on membranes.


## 1. Introduction

Biofilm formation is generally undesirable in the food industry, because it represents a potential source of microbial contamination and spoilage enzymes affecting food quality (Teh et al., 2014), and affect processing efficiency (Seale, Bremer, Flint, Brooks, & Palmer, 2015a). In the dairy industry, processing equipment such as filtration systems are used in a continuous mode for extended operation times (>20 h). These conditions are particularly susceptible to biofilm formation (Anand, Singh, Avadhanula, & Marka, 2014). Indeed, the filtration environment is steady (which is essential for biofilm formation, even in the presence of turbulence), offers a constant supply of nutrients (proteins, sugars and minerals) and is at a temperature suitable for microbial growth (between 10 °C and 50 °C) (Tang, Flint, Bennett, Brooks, & Zain, 2015). Filtration membranes are further susceptible to biofilm formation since mechanical cleaning is not possible in this closed environment and the constant convective flow through the membrane, due to transmembrane pressure, may facilitate bacterial adhesion to the membrane surface (Choi, Zhang, Dionysiou, Oerther, & Sorial, 2005; Ridgway et al., 1999; Simões, Simões, & Vieira, 2010).

The main strategy used to control biofouling in the dairy industry is to clean membranes daily with a clean-in-place (CIP) system, circulating acid and alkaline solutions at the membrane surface following a filtration time of up to 24 h (Anand et al., 2014; Berg et al., 2014). This cleaning process restores the membrane permeation flux (Trägårdh, 1989). However, traditional CIP may not remove or kill bacteria cells adhered on stainless steel or filtration membrane surfaces (Bénézech & Faille, 2018; Marka & Anand, 2017; İpek & Zorba, 2018), even with optimised CIP protocols (Kumari & Sarkar, 2014). At the laboratory scale, innovative biofilm removal or control strategies dedicated to the membrane separation industry have been suggested: the use of ozonised water (Henderson et al., 2016), surfactants (Hijnen et al., 2012) or specific enzymes (Khan et al., 2014; Tang, Flint, Bennett, & Brooks, 2010), quorum quenchers (Yeon et al., 2009), quorum quenching bacteria (Kampouris et al., 2018; Oh et al., 2017) or bioengineered biofilms (Wood et al., 2016). The use of specific enzymes is actually recommended in the industry to control short-term issues related to biofouling, but are still inefficient in a long-term purposes (Simões et al., 2010).

The complete removal of biofilms from filtration membranes may not be possible (Bucs et al., 2018; Simões et al., 2010). Instead of eradicating them from filtration systems, it appears more realistic to delay biofilm formation (Bucs et al., 2018), or to reduce their negative impact through the selection of operational parameters

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that limit the growth of problematic bacteria such as those that are thermoresistant. Recently, the chemistry of the membrane material was found to affect the persistence of a problematic spore-former (*Bacillus* sp.) on filtration membranes following whey ultrafiltration (UF) (Chamberland et al., 2017a). Lowering the temperature of the feed also appears a good strategy since it reduced the incidence of biofouling on reverse osmosis membranes in the desalination industry (Farhat, Vrouwenvelder, Van Loosdrecht, Bucs, & Staal, 2016), and the adhesion of *Streptococcus* sp. on PES membranes during milk or whey UF (Chamberland, Lessard, Doyen, Labrie, & Pouliot, 2017b).

Two common feed temperatures are used during UF of dairy fluids. Although higher feed temperatures (i.e., 50 °C) provide higher permeation fluxes (Méthot-Hains et al., 2016; St-Gelais, Haché, & Gros-Louis, 1992; Yan, Hill, & Amundson, 1979), better microbial quality of the retentate is expected when filtration is performed at a colder temperature (<20 °C) due to a lower number of bacteria in it (Kapsimalis & Zall, 1981; Maubois & Mocquot, 1975; Pompei, Resmini, & Peri, 1973). However, when filtering fluids with fat or concentrating milk at a high concentration factor, a higher feed temperature is required to reduce the viscosity of the feed, even if the growth of thermophilic bacteria can occur (Maubois & Mocquot, 1975; Seale et al., 2015b). Consequently, this study was needed to document the composition of bacterial community forming on dairy filtration membranes at different time points and temperatures. It permitted to assess if the formation of biofilm on membranes is possible in a single processing day at cold temperature (15 °C), and to determine a safe processing time at hot temperature (50 °C) to avoid the exponential growth phase of bacteria adhering on membranes in contact with pasteurised milk. Bacteria were characterised by a metabarcoding approach and quantified by real-time PCR (qPCR), from membranes sampled in a biofilm reactor designed to mimic a filtration system.

## 2. Material and methods

### 2.1. Milk source

A different batch lot of raw milk (temperature <4 °C, pH between 6.65 and 6.75) was obtained from a local dairy prior each experiment. Milk was skimmed (<0.01% fat) and pasteurised (75 °C for 16 s) as previously described (Chamberland et al., 2017b). The pasteurised milk was directly collected in a sterilised 20 L feed tank (Cole-Parmer, Montreal, QC, Canada) called the milk tank, and was stored at 4 °C. The time between the pasteurisation and the beginning of each experiment was 12 h or less.

### 2.2. Biofilm reactor assembly and operation

A biofilm reactor (CBR 90, BioSurfaces technologies corporation, Bozeman, MN, USA) that mimics the filtration environment was used to study the attachment of bacteria on 10 kDa polyethersulfone (PES) UF membranes (ST membrane, Synder, Vacaville, CA, USA) (Fig. 1). Membrane coupons measuring 6 $cm^2$ were fixed on eight membrane holders, between the holders and the restraining frames specifically designed for the biofilm reactor. The eight holders are placed in such a way in the bioreactor that the flow condition is identical for each membrane (Fig. 1B). The milk tank was always maintained at 4 °C in an incubator (MIR-153, Sanyo, Osaka, Japan) throughout the biofilm development experiments (Fig. 1). A peristaltic pump (Masterflex, Model 7518-00, Barnant Company, Barrington, IL, USA) continuously transferred milk from the milk tank to the biofilm reactor through silicone tubing (Tubing M-Flex L/S 16, Cole-Parmer, Montreal, Canada) at a constant flow rate of 0.25 mL $s^{-1}$ (flow rate value between those of Bremer, Fillery, and McQuillan (2006) and Dufour, Simmonds, and Bremer (2004)). The stirring rate inside the reactor was 180 rpm, as described by Tang, Flint, Brooks, and Bennett (2009), to generate a flow regime considered as turbulent according to Buckingham-Meyer, Goeres, and Hamilton (2007).

The biofilm formation experiments were performed at 15 °C and 50 °C. The reactor was maintained at 15 ± 1 °C by a cold water bath or at 50 ± 1 °C by a stirring hot plate (Troemner, LLC; Thorofare, NJ, USA) connected with a sterile temperature sensor inside the biofilm reactor. Experiments were performed in triplicate (n = 3) for each temperature condition.

Efforts were made to mimic as much as possible the filtration system environment by operating the biofilm reactor in the continuous mode, at representative operating temperatures, with the highest level of turbulence possible, and with pasteurised milk having its natural microflora (thermoresistant bacteria and environmental contaminants). However, the turbulent flow regime and shear stress at membrane's surface in a filtration system could not be reproduced using the bioreactor.

### 2.3. Bioreactor preparation

Prior to each experiment, the biofilm reactor and its different parts (tanks and tubing) were autoclaved at 121 °C for 15 min. Membrane coupons were then fixed aseptically on the membrane holders and a conventional CIP of the system (conditioning step) was performed as recommended by the membrane manufacturer. Briefly, the CIP procedure consisted of alkaline (pH 10.5), acid (pH

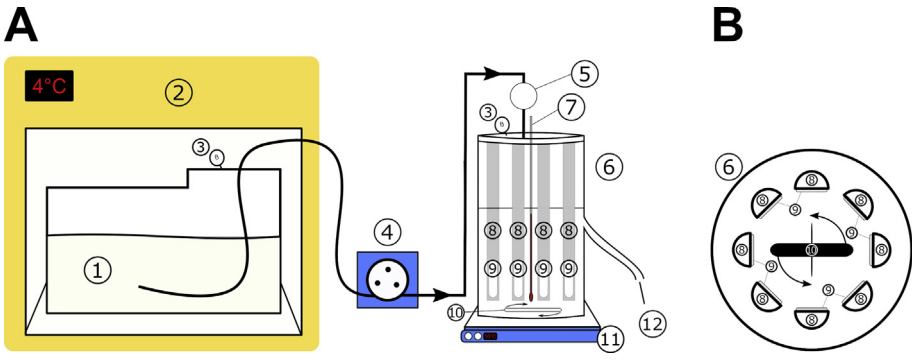


**Fig. 1.** Biofilm reactor (A) consisting of a feed tank (1) maintained at 4 °C in an incubator (2), bacterial air vents (3), a peristaltic pump (4), a flow break (5), a CDC biofilm reactor (6), a temperature sensor (7), eight membrane holder rods (8) and eight membranes (9), a stir bar (10), a stirring/hot plate (11) and a waste collector (12), and (B) biofilm reactor seen from the top.

2.0) and chlorinated alkaline (pH 10.5, 150 ppm of free chlorine) cleaning steps, executed consecutively. Cleaning solutions were prepared with commercial chemicals: Membra-base 210 (Sani-Marc, Victoriaville, QC, Canada), Ultrasil 75 (Ecolab, Saint Paul, MN, USA) and Chloreco (Sani-Marc). Each cleaning step was performed for 30 min at 50 °C. A rinsing step with deionised water at 50 °C preceded and followed each individual step of the CIP. The stirring rate of 180 rpm was maintained in the biofilm reactor during the cleaning procedure. A cleaning step under the same conditions was also performed at the end of each experiment.

### 2.4. Membrane and milk sampling

During experiments performed at 15 °C, membranes were collected with a sterile scalpel after the membrane conditioning step (0 h), after 20 h, 24 h, 28 h, 32 h, 36 h, 48 h and after the final cleaning step at the end of the experiment. Considering the shorter development time of bacteria at warmer temperatures, the membranes were collected in shorter intervals of time at 50 °C: after the membrane conditioning step, after 7.5 h, 10 h, 12.5 h, 15 h, 20 h and after the last cleaning step. Prior to being stored at −80 °C until DNA extraction, membranes were gently rinsed in a sterile phosphate buffered saline (PBS) solution (pH 7.4) to remove planktonic bacteria from membranes (Anand, Hassan, & Avadhanula, 2012).

Milk inside the biofilm reactor was sampled at the same time points as the membranes. For each sampling time, three samples of 2 mL were pelleted and stored at −80 °C until DNA extraction.

### 2.5. Targeted genomic analysis of the bioreactor microbiome

#### 2.5.1. Genomic DNA extraction

For both temperature conditions, genomic DNA, which contains DNA from dead and viable cells, was extracted in duplicate from the milk and the membrane samples as described previously (Chamberland, Lessard, Doyen, Labrie, & Pouliot, 2017c), except that sodium acetate (pH 5.2) at a final concentration of 0.3 M was used to precipitate the DNA (Sambrook & Russell, 2001).

#### 2.5.2. High-throughput sequencing and bioinformatics

Amplicon sequencing targeting the V6–V8 region of the 16S ribosomal RNA (rRNA) gene was performed on a Miseq sequencer at the Institut de Biologie Intégrative et des Systèmes (Université Laval, Québec, QC, Canada). Two replicates for each temperature condition were used for the sequencing step. Raw demultiplexed paired-end reads of each replicate were deposited in the GenBank database under the accession number SRP140914 (first replicate, 3,285,298 raw demultiplexed reads) and SRP150623 (second replicate, 3,169,154 raw demultiplexed reads). The computations of the reads of each replicate were processed separately, as previously described (Chamberland et al., 2017a), with the modifications described next. Computations were done with the pipeline FROGS from the Galaxy portal of the INRA MIGALE Bioinformatics platform (Jouy-en-Josas Cedex, France; Escudié et al., 2017; Goecks et al., 2010). Following reads preprocessing (quality filter, read trimming and read assembly), contiged reads were clustered with the Swarm clustering method using the denoising clustering step and the suggested aggregation distance of 3 (Mahé, Rognes, Quince, de Vargas, & Dunthorn, 2014). A first chimera filter was performed with VSEARCH (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Operational taxonomic units (OTU) with abundance lower than 0.005% were removed (Bokulich et al., 2013). The SILVA database (release 132, December 13, 2017) was used to perform the OTU affiliations. Each OTU was manually inspected a second time to remove undetected chimeras. The final OTU tables obtained for each replicate were finally merged by taking the sum of the absolute abundance of each taxon. Ecological metrics (alpha- and beta-diversity) were computed with the Mothur pipeline (v1.35.1) (Schloss et al., 2009), as previously described (Chamberland et al., 2017c).

#### 2.5.3. Quantification of bacterial growth

The number of 16S rRNA gene copies found on membranes and in milk at each time point were quantified by qPCR as described previously (Chamberland et al., 2017a).

### 2.6. Statistical analysis

As mentioned, the experimental design was repeated three times for each temperature. A one-way analysis of variance (ANOVA) of the number of 16S rRNA gene copies variable was performed with RStudio (v.1.0.136) using the package agricolae (v1.2-8). The analysis was performed separately for membrane and milk samples. The number of gene copies among samples were considered significantly different with a $p$-value smaller than 0.05 (Fisher's least significant difference test).

## 3. Results

### 3.1. Quantification of the number of 16S rRNA gene copies on membranes

Changes in bacterial communities in milk and on membranes were studied as function of time for two temperatures, 15 °C and 50 °C (Fig. 2). The bacterial communities observed on membranes were significantly different depending on whether the membranes were used at 15 °C or 50 °C (Fig. 2A). At 50 °C, a significant increase in the number of 16S rRNA gene copies was observed throughout the experiment ($P < 0.05$, Fig. 2A). The number of gene copies per $cm^2$ increased significantly from 3.21 ± 0.12 $\log_{10}$ after the first cleaning to 5.40 ± 0.97 $\log_{10}$ gene copies per $cm^2$ ($P < 0.05$) after 7.5 h, finally reaching a maximum of 8.83 ± 1.58 $\log_{10}$ gene copies per $cm^2$ after 15 h ($P < 0.05$, Fig. 2A). Following the cleaning step performed after 20 h of operation, the number of gene copies (5.57 ± 3.38 $\log_{10}$ gene copies per $cm^2$) was still significantly higher than at the beginning of the process (3.21 ± 0.12 $\log_{10}$ gene copies per $cm^2$, $P < 0.05$, Fig. 2A).

At 15 °C, the number of 16S rRNA gene copies did not increase significantly on the membranes throughout the 48 h experiment. It varied from 3.31 ± 0.24 $\log_{10}$ gene copies per $cm^2$ after the first cleaning to 3.86 ± 0.58 $\log_{10}$ gene copies per $cm^2$ after 48 h ($P > 0.05$, Fig. 2A). The initial count was not significantly different from the final count measured after the last cleaning (3.48 ± 0.58, $P > 0.05$, Fig. 2A).

### 3.2. Composition of bacterial communities formed on membranes

The temperature of the feed also affected the diversity and richness of bacteria observed on membranes. Globally, communities formed at 15 °C had more diversity and richness (Table 1). For example, the diversity index (Inverse Simpson) was between 1.00 and 6.57 on membranes operated at 50 °C while it was between 8.35 and 20.58 at 15 °C (Table 1). In the same vein, membranes operated at 50 °C had a lower richness, estimated between 7.84 and 67.83 species, compared with between 63.51 and 180.70 at 15 °C (Chao index, Table 1).

Communities formed at 50 °C were dominated by the Bacilli class (more than 60.15% from 7.5 h, Supplementary material Table S1). In contrast, those formed at the colder temperature were composed of Actinobacteria (13.46%–26.26%), Bacteroidia (12.53%–27.00%), Bacilli (8.01%–20.69%), α- and γ-Proteobacteria

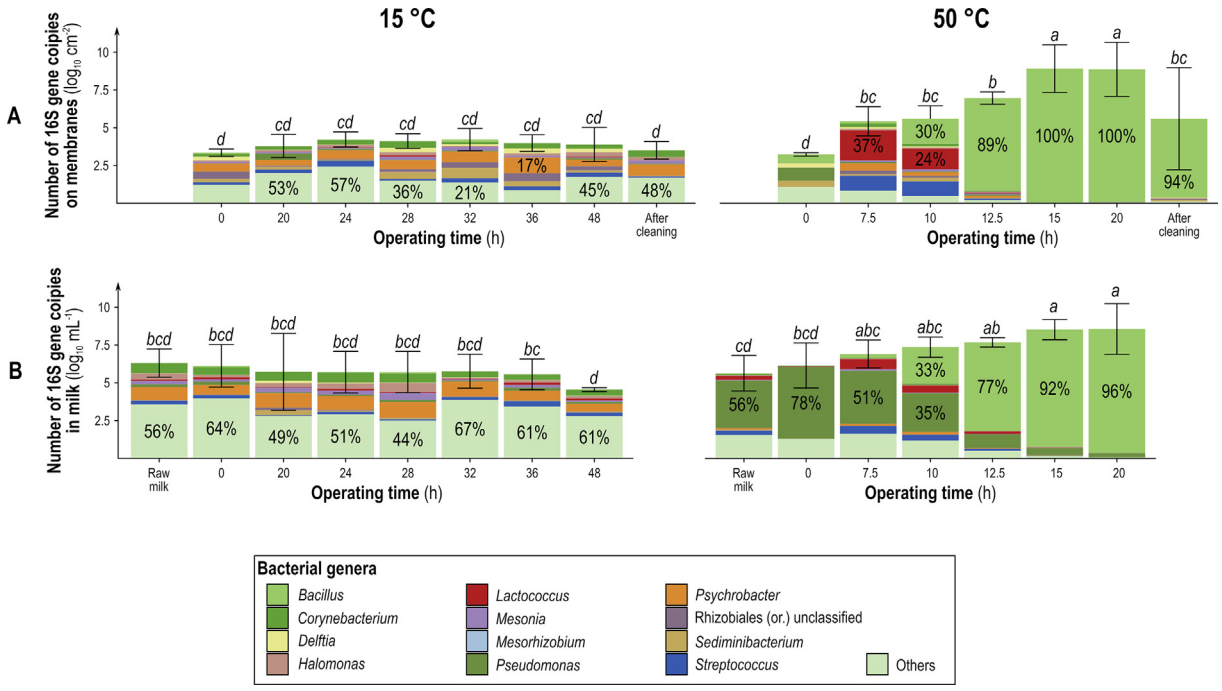


**Fig. 2.** Portrait of bacterial communities formed on membranes (A) and bacteria found in milk (B) inside the bioreactor at 15 °C or 50 °C. Bars with the same letter do not have a significantly different gene copy number (Fischer's least significant difference test, $P > 0.05$, n = 3). The percentages indicate the proportion of each genus.

**Table 1**
Ecological metrics of communities formed on membranes at 15 °C and at 50 °C.[a]

| Temperature (°C) | Time (h) | $S_{obs}$ | Diversity index (Inverse Simpson) | Richness index (Chao) |
|---|---|---|---|---|
| 15 | 0 | 149 | 13.69 | 162.42 |
| | 20 | 125 | 20.58 | 132.18 |
| | 24 | 142 | 19.44 | 149.99 |
| | 28 | 88 | 12.78 | 94.49 |
| | 32 | 60 | 8.35 | 63.51 |
| | 36 | 98 | 11.69 | 109.44 |
| | 48 | 148 | 20.11 | 172.40 |
| | After cleaning | 172 | 12.70 | 180.70 |
| 50 | 0 | 18 | 6.57 | 18.00 |
| | 7.5 | 62 | 5.35 | 67.83 |
| | 10 | 61 | 5.41 | 65.34 |
| | 12.5 | 50 | 1.27 | 57.87 |
| | 15 | 15 | 1.01 | 25.41 |
| | 20 | 5 | 1.00 | 7.84 |
| | After cleaning | 25 | 1.14 | 27.61 |

[a] Abbreviation: $S_{obs}$, number of OTU observed. For diversity and richness indices, each sample had sequencing coverage of greater than 99%; analyses were performed on rarefied samples containing 16,536 sequence reads.

(2.77%–23.94% and 21.80%–35.27%, respectively) classes (Supplementary material Table S2). Low proportions of the Clostridia class were also observed (0.04–4.32%) at 15 °C (Supplementary material Table S2), while this class was not present at 50 °C, or in trace concentrations (0.67% after 7.5 h, Supplementary material Table S1).

Of the bacterial genera observed on membranes, the *Lactococcus* genus was the most abundant during the first part of the experiment performed at 50 °C, representing 37.18% of the community on the membrane sampled after 7.5 h (Fig. 2A). However, at this temperature, exponential growth of *Bacillus* spp. occurred, presumably primarily *B. licheniformis* based on a local BLAST alignment, from 10 h to 15 h of operation. The number of 16S gene copies affiliated to the *Bacillus* genus increased from 1.66 to 6.14 $\log_{10}$ gene copies per $cm^2$ between these sampling times (Fig. 2A). The *Bacillus* genus reached stationary phase at 15 h of operation and persisted on membranes, even after cleaning.

At 15 °C, psychrotrophic (cold-tolerant) bacterial genera were observed on membranes, such as *Corynebacterium* (ratios between 3.51 and 12.28%), *Halomonas* (ratios between 1.10% and 7.67%), *Pseudomonas* (ratios between 0.09 and 11.65%) or *Psychrobacter* (ratios between 10.26% and 26.75%) (Fig. 2A and Table S2). *Psychrobacter* was the most abundant genus, however, no one genus was dominant, and no exponential growth was observed throughout the 48 h-experiments (Fig. 2A). The *Bacillus* genus was only found in low ratios (0.19%–3.92%) at this temperature (Fig. 2A).

### 3.3. Changes in bacterial diversity in milk inside the bioreactor

As on the membranes, there were few variations in the proportions of bacterial OTU in milk circulating in the bioreactor at 15 °C. A decrease in the number of 16S rRNA gene copies was suspected, from 6.08 ± 1.41 $\log_{10}$ gene copies per mL at the beginning of the experiment to 4.52 ± 0.12 $\log_{10}$ gene copies per mL

after 48 h of operation, but the difference was not significant ($P > 0.05$, Fig. 2B). At 50 °C, an increase in *Bacillus* spp. was observed in milk, similar to the finding with the membranes, but an increase in the number of 16S gene copies was only significant after 15 h of operation, where the number of copies reached 8.49 ± 0.67 $\log_{10}$ gene copies per mL ($P < 0.05$, Fig. 2B).

## 4. Discussion

### 4.1. Operating at 50 °C, a race against spore-former bacteria

The most important predictable issues resulting from UF of dairy fluid at 50 °C is the formation of biofilms composed of thermophilic bacteria on UF membranes during the process (Anand et al., 2014; Burgess, Lindsay, & Flint, 2010; Pompei et al., 1973), and the subsequent contamination of the feed, as seen in milk circulating inside the bioreactor (Fig. 2B). These bacteria, including the commonly found *Anoxybacillus flavithermus*, *Geobacillus* spp. and *Bacillus* spp. are generally not pathogenic, but their biofilm-forming and spore-forming abilities make them extremely difficult to remove or kill using conventional heat treatments, and their heat-stable enzymes may also affect the quality of dairy products (Burgess et al., 2010; Cho et al., 2018; Sadiq et al., 2017). Complete CIP may be sufficient to kill them on stainless steel, which support high temperature cleaning (Parkar, Flint, & Brooks, 2004). However, on weaker materials such as polymeric filtration membranes, it was observed that biofilms of *Bacillus* spp. are one of the most resistant to CIP solutions (Anand & Singh, 2013).

This study determined that a 15 h run at 50 °C (time needed to reach a stationary phase on membranes) allows mature and saturated biofilm composed of spore-former bacteria (*Bacillus* spp.) to form on a cleaned membrane during its first use. The *Bacillus* genus entered exponential growth phase after approximately 10 h and grew significantly in a short period of time, as found in other studies with doubling times corresponding to 0.25 h and to 0.52 h at this temperature (Burgess et al., 2010; Dufour et al., 2004; Gauvry et al., 2017). In comparison, *Streptococcus thermophilus* biofilms may be formed in 6 h in the regeneration section of a pasteuriser, at a temperature between 30 °C and 40 °C (Bouman, Lund, Driessen, & Schmidt, 1982; Knight, Nicol, & McMeekin, 2004), while the contamination of thermophilic spore-forming bacteria appears from 9 h during an evaporation process (Murphy, Lynch, & Kelly, 1999; Scott, Brooks, Rakonjac, Walker, & Flint, 2007).

*Bacillus* spp. have been shown to persist on reverse osmosis membranes in contact with whey for 24 h following every step of a CIP (Anand & Singh, 2013). According to the findings of Keren, Kaldalu, Spoering, Wang, and Lewis (2004), the number of persistent cells of *Bacillus* would have increased in this study since the biofilm reached stationary phase. The high ratio of *Bacillus* spp. observed on the cleaned membrane following the 50 °C experiment (Fig. 2A) may thus represent viable bacteria not removed by the CIP. However, since the genomic approach selected for this study also detects DNA from dead cells, additional work complementary to those of Anand and Singh (2013) is needed to confirm the metabolic state of bacteria on membranes following a CIP. This work will permit to determine if bacteria detected on membranes are alive and contribute actively to the formation of biofilms during the following process, or if they are dead, and rather contribute to the formation of biofilms by enhancing further bacterial adhesion on membranes.

### 4.2. Cold temperature as a key parameter to delay biofilm formation

As mentioned previously, there is a trend in the dairy industry to perform continuous unit operations such as UF at colder temperatures (Tang et al., 2009) to provide dairy products with a better microbial quality, even if this reduces membrane performance because of higher milk viscosity (Kapsimalis & Zall, 1981; Yan et al., 1979).

As mentioned by Yuan, Burmølle, Sadiq, Wang, and He (2018), few studies have looked at biofilm formation assays at cold temperatures and whether some psychrotrophic bacteria found in raw milk, such as *Pseudomonas fluorescens*, have increased biofilm-forming abilities at colder temperatures (Aswathanarayan & Vittal, 2014). Indeed, biofilms have already been found on dairy industrial filtration membranes operated around 15 °C with pasteurised milk (Chamberland et al., 2017c; Tang et al., 2009).

In this study, membranes operated at cold temperature were colonised with bacteria such as *Pseudomonas*, *Lactococcus* and *Sediminibacterium*, as observed previously following 5 h UF at 10 °C in a model cross-flow filtration system (Chamberland et al., 2017b). However, this 48 h experiment did not provide sufficient evidence for biofilm formation or for growth on the membranes since the numbers of 16S rRNA gene copies on membranes did not differ significantly across the times sampled ($P > 0.05$, Fig. 2A). At 15 °C, both psychrotrophic and mesophilic bacteria can grow in the dairy environment (Seale et al., 2015a). However, mesophilic bacteria such as lactic acid bacteria have a slow growth rate at this temperature (Adamberg, Kask, Laht, & Paalme, 2003) and psychrotrophic bacteria from raw milk that survive pasteurisation are scarce (Coghill, 1982; Quigley et al., 2013a). Even if it was demonstrated that a psychrotrophic genus such as *Pseudomonas* was more resistant to heat treatment than previously stated (Quigley et al., 2013a), most of the psychrotrophic bacteria found in pasteurised milk originate through post-contamination from the water source or the plant environment (Chamberland et al., 2017b; Coghill, 1982; Quigley et al., 2013b).

Since water used to perform the cleaning cycle of the pasteuriser and the membranes in this study was demineralised and of high purity, the number of metabolically active bacteria in the pasteurised milk and adhering to membranes was probably very low. The biofilm reactor and its components were also autoclaved prior to each experiment, which limited the possibility of environmental contamination of milk. Habimana, Heffernan, and Casey (2017) formed *P. fluorescens* biofilms on nanofiltration membranes in less than 48 h at 20 °C, but their inoculation rate was higher. Extended membrane usage time is required to determine the time needed for psychrotrophic biofilms to be formed.

In this study, communities formed at 15 °C did not have a detectable exponential growth phase. Their tolerance to the CIP cycle was possibly lower than communities formed at 50 °C, as revealed by a similar number of 16S rRNA gene copies at the beginning and at the end of the 48 h experiment (Fig. 2A) (Anand & Singh, 2013; Keren et al., 2004). Since the membrane cleaning is generally performed at 50 °C, it would be interesting to determine if the higher thermal stress imposed by the CIP on communities formed at 15 °C could be a long-term advantage to reduce the biofilm formation rate on membranes. The destabilisation of bacteria through temperature changes permitted control of *S. thermophilus* biofilms in the regenerative section of pasteurisers (Knight et al., 2004). This stress, imposed with a multi-temperature filtration process, may contribute to reduce biofilm formation on filtration membranes.

### 4.3. The representativeness of the biofilm reactor

Necessarily, results obtained from a biofilm reactor in this study did not reflect perfectly the reality of industrial filtration systems. Indeed, according to observations made in the desalination industry (reverse osmosis membranes), a higher flow velocity in

filtration system or the presence of a concentration polarisation at the membrane surface may affect the time needed to form biofilms on membranes (Suwarno et al., 2014). On the other hand, the use of a biofilm reactor helped to generate membrane samples at different time points without affecting the retentate flow, and to determine more precisely the impact of the temperature in a controlled environment. In comparison with other studies made previously, the ratio of the bacteria among communities forming on membranes in model filtration system or in the biofilm reactor were different (Chamberland et al., 2017a,b). However, common dairy bacterial genus such as *Bacillus* spp., *Lactococcus* spp., *Pseudomonas* spp. or *Streptococcus* spp. were observed in both systems (Chamberland et al., 2017a,b).

## 5. Conclusions

The temperature of the feed, as shown in this study, is a crucial parameter to consider for preventing biofilm formation on membranes. Indeed, at the industrial scale, the conclusions presented here cannot be taken as an argument to perform cold milk UF over 48 h. However, this study revealed that in a system wherein bacterial contaminants are well controlled (system perfectly cleaned and cleaned with good quality water), the biofilm formation rate on membranes is considerably slower at 15 °C than at 50 °C. Indeed, in a system were the daily cleaning procedures are rigorously performed, biofouling may not be an issue at 15 °C. However, as noted, additional work is needed to determine how psychrotrophic biofilms persist on filtration membranes and affect cleaning efficiency in real filtration systems. Industrially, if the feed viscosity does not permit operation at cold temperatures, or if industrial installations are not available, filtration at 50 °C may be mandatory. Then, the operating time should be limited to 10 h to avoid the exponential growth of thermoresistant bacteria such as *Bacillus* sp. on membranes.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2019.02.005.

## References

Adamberg, K., Kask, S., Laht, T., & Paalme, T. (2003). The effect of temperature and pH on the growth of lactic acid bacteria: A pH-auxostat study. *International Journal of Food Microbiology, 85*, 171–183.

Anand, S., Hassan, A., & Avadhanula, M. (2012). The effects of biofilms formed on whey reverse osmosis membranes on the microbial quality of the concentrated product. *International Journal of Dairy Technology, 65*, 451–455.

Anand, S., & Singh, D. (2013). Resistance of the constitutive microflora of biofilms formed on whey reverse-osmosis membranes to individual cleaning steps of a typical clean-in-place protocol. *Journal of Dairy Science, 96*, 6213–6222.

Anand, S., Singh, D., Avadhanula, M., & Marka, S. (2014). Development and control of bacterial biofilms on dairy processing membranes. *Comprehensive Reviews in Food Science and Food Safety, 13*, 18–33.

Aswathanarayan, J. B., & Vittal, R. R. (2014). Attachment and biofilm formation of *Pseudomonas fluorescens* PSD4 isolated from a dairy processing line. *Food Science and Biotechnology, 23*, 1903–1910.

Bénézech, T., & Faille, C. (2018). Two-phase kinetics of biofilm removal during CIP. Respective roles of mechanical and chemical effects on the detachment of single cells vs cell clusters from a *Pseudomonas fluorescens* biofilm. *Journal of Food Engineering, 219*, 121–128.

Berg, T. H. A., Knudsen, J. C., Ipsen, R., Van Den Berg, F., Holst, H. H., & Tolkach, A. (2014). Investigation of consecutive fouling and cleaning cycles of ultrafiltration membranes used for whey processing. *International Journal of Food Engineering, 10*, 367–381.

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., et al. (2013). Attachment and biofilm formation of *Pseudomonas fluorescens* PSD4 isolated from a dairy processing line. *Nature Methods, 10*, 57–59.

Bouman, S., Lund, D. B., Driessen, F. M., & Schmidt, D. G. (1982). Growth of thermoresistant streptococci and deposition of milk constituents on plates of heat exchangers during long operating times. *Journal of Food Protection, 9*, 788–883.

Bremer, P. J., Fillery, S., & McQuillan, A. J. (2006). Laboratory scale clean-in-place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *International Journal of Food Microbiology, 106*, 254–262.

Buckingham-Meyer, K., Goeres, D. M., & Hamilton, M. A. (2007). Comparative evaluation of biofilm disinfectant efficacy tests. *Journal of Microbiological Methods, 70*, 236–244.

Bucs, S. S., Farhat, N., Kruithof, J. C., Picioreanu, C., van Loosdrecht, M. C. M., & Vrouwenvelder, J. S. (2018). Review on strategies for biofouling mitigation in spiral wound membrane systems. *Desalination, 434*, 189–197.

Burgess, S. A., Lindsay, D., & Flint, S. H. (2010). Thermophilic bacilli and their importance in dairy processing. *International Journal of Food Microbiology, 144*, 215–225.

Chamberland, J., Beaulieu-Carbonneau, G., Lessard, M.-H., Labrie, S., Bazinet, L., Doyen, A., et al. (2017a). Effect of membrane material chemistry and properties on biofouling susceptibility during milk and cheese whey ultrafiltration. *Journal of Membrane Science, 542*, 208–216.

Chamberland, J., Lessard, M.-H., Doyen, A., Labrie, S., & Pouliot, Y. (2017b). Biofouling of ultrafiltration membranes by dairy fluids: Characterization of pioneer colonizer bacteria using a DNA metabarcoding approach. *Journal of Dairy Science, 100*, 981–990.

Chamberland, J., Lessard, M.-H., Doyen, A., Labrie, S., & Pouliot, Y. (2017c). A sequencing approach targeting the 16S rRNA gene unravels the biofilm composition of spiral-wound membranes used in the dairy industry. *Dairy Science & Technology, 96*, 827–843.

Choi, H., Zhang, K., Dionysiou, D. D., Oerther, D. B., & Sorial, G. A. (2005). Effect of permeate flux and tangential flow on membrane fouling for wastewater treatment. *Separation and Purification Technology, 45*, 68–78.

Cho, T. J., Kim, H. W., Kim, N. H., Park, S. M., Kwon, J. I., Kim, J., et al. (2018). New insights into the thermophilic spore-formers in powdered infant formula: Implications of changes in microbial composition during manufacture. *Food Control, 92*, 464–470.

Coghill, D. (1982). Studies on thermoduric psychrotrophic bacteria in south east Queensland dairy products. *Australian Journal of Dairy Technology, 37*, 147–148.

Dufour, M., Simmonds, R. S., & Bremer, P. J. (2004). Development of a laboratory scale clean-in-place system to test the effectiveness of "natural" antimicrobials against dairy biofilms. *Journal of Food Protection, 67*, 1438–1443.

Escudié, F., Auer, L., Bernard, M., Mariadassou, M., Cauquil, L., Vidal, K., et al. (2017). FROGS: Find, rapidly, OTUs with galaxy solution. *Bioinformatics, 34*, 1287–1294.

Farhat, N. M., Vrouwenvelder, J. S., Van Loosdrecht, M. C. M., Bucs, S. S., & Staal, M. (2016). Effect of water temperature on biofouling development in reverse osmosis membrane systems. *Water Research, 103*, 149–159.

Gauvry, E., Mathot, A. G., Leguérinel, I., Couvert, O., Postollec, F., Broussolle, V., et al. (2017). Knowledge of the physiology of spore-forming bacteria can explain the origin of spores in the food environment. *Research in Microbiology, 168*, 369–378.

Goecks, J., Nekrutenko, A., Taylor, J., Afgan, E., Ananda, G., Baker, D., et al. (2010). Galaxy: A comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biology, 11*, R86.

Habimana, O., Heffernan, R., & Casey, E. (2017). Nanofiltration-induced cell death: An integral perspective of early stage biofouling under permeate flux conditions. *Journal of Membrane Science, 541*, 93–100.

Henderson, R., Gandhi, G., Sevart, N., Gragg, S., Phebus, R., & Amamcharla, J. K. (2016). Use of ozonated water in removing *Bacillus cereus* biofilms from the dairy membranes. *Journal of Animal Science, 94*(S5), 342.

Hijnen, W. A. M., Castillo, C., Brouwer-Hanzens, A. H., Harmsen, D. J. H., Cornelissen, E. R., & van der Kooij, D. (2012). Quantitative assessment of the efficacy of spiral-wound membrane cleaning procedures to remove biofilms. *Water Research, 46*, 6369–6381.

İpek, D., & Zorba, N. N. D. (2018). Microbial load of white cheese process lines after CIP and COP: A case study in Turkey. *LWT - Food Science and Technology, 90*, 505–512.

Kampouris, I. D., Karayannakidis, P. D., Banti, D. C., Sakoula, D., Konstantinidis, D., Yiangou, M., et al. (2018). Evaluation of a novel quorum quenching strain for MBR biofouling mitigation. *Water Research, 143*, 56–65.

Kapsimalis, D. J., & Zall, R. R. (1981). Ultrafiltration of skim milk at refrigerated temperatures. *Journal of Dairy Science, 64*, 1945–1950.

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., & Lewis, K. (2004). Persister cells and tolerance to antimicrobials. *FEMS Microbiology Letters, 230*, 13–18.
Khan, M., Danielsen, S., Johansen, K., Lorenz, L., Nelson, S., & Camper, A. (2014). Enzymatic cleaning of biofouled thin-film composite reverse osmosis (RO) membrane operated in a biofilm membrane reactor. *Biofouling, 30*, 153–167.
Knight, G. C., Nicol, R. S., & McMeekin, T. A. (2004). Temperature step changes: A novel approach to control biofilms of *Streptococcus thermophilus* in a pilot plant-scale cheese-milk pasteurisation plant. *International Journal of Food Microbiology, 93*, 305–318.
Kumari, S., & Sarkar, P. K. (2014). In vitro model study for biofilm formation by *Bacillus cereus* in dairy chilling tanks and optimization of clean-in-place (CIP) regimes using response surface methodology. *Food Control, 36*, 153–158.
Mahé, F., Rognes, T., Quince, C., de Vargas, C., & Dunthorn, M. (2014). Swarm: Robust and fast clustering method for amplicon-based studies. *PeerJ, 2*, e593.
Marka, S., & Anand, S. (2017). Feed substrates influence biofilm formation on reverse osmosis membranes and their cleaning efficiency. *Journal of Dairy Science, 101*, 84–95.
Maubois, J. L., & Mocquot, G. (1975). Application of membrane ultrafiltration to preparation of various types of cheese. *Journal of Dairy Science, 58*, 1001–1007.
Méthot-Hains, S., Benoit, S., Bouchard, C., Doyen, A., Bazinet, L., & Pouliot, Y. (2016). Effect of transmembrane pressure control on energy efficiency during skim milk concentration by ultrafiltration at 10 and 50°C. *Journal of Dairy Science, 99*, 8655–8664.
Murphy, P. M., Lynch, D., & Kelly, P. M. (1999). Growth of thermophilic spore forming bacilli in milk during the manufacture of low heat powders. *International Journal of Dairy Technology, 52*, 45–50.
Oh, H. S., Tan, C. H., Low, J. H., Rzechowicz, M., Siddiqui, M. F., Winters, H., et al. (2017). Quorum quenching bacteria can be used to inhibit the biofouling of reverse osmosis membranes. *Water Research, 112*, 29–37.
Parkar, S. G., Flint, S. H., & Brooks, J. D. (2004). Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *Journal of Applied Microbiology, 96*, 110–116.
Pompei, C., Resmini, P., & Peri, C. (1973). Skim milk protein recovery and purification by ultrafiltration, Influence of temperature on permeation rate and retention. *Journal of Food Science, 38*, 867–870.
Quigley, L., McCarthy, R., O'Sullivan, O., Beresford, T. P., Fitzgerald, G. F., Ross, R. P., et al. (2013a). The microbial content of raw and pasteurized cow milk as determined by molecular approaches. *Journal of Dairy Science, 96*, 4928–4937.
Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., et al. (2013b). The complex microbiota of raw milk. *FEMS Microbiology Reviews, 37*, 664–698.
Ridgway, H., Ishida, K., Rodriguez, G., Safarik, J., Knoell, T., & Bold, R. (1999). Biofouling of membranes: Membrane preparation, characterization, and analysis of bacterial adhesion. *Methods in Enzymology, 310*, 463–494.
Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ, 4*, e2584.
Sadiq, F. A., Flint, S., Yuan, L., Li, Y., Liu, T., & He, G. (2017). Propensity for biofilm formation by aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. *International Journal of Food Microbiology, 262*, 89–98.
Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: A laboratory manual* (3rd ed.). New York: CSHL Press.
Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology, 75*, 7537–7541.
Scott, S. A., Brooks, J. D., Rakonjac, J., Walker, K. M. R., & Flint, S. H. (2007). The formation of thermophilic spores during the manufacture of whole milk powder. *International Journal of Dairy Technology, 60*, 109–117.
Seale, B., Bremer, P., Flint, S., Brooks, J., & Palmer, J. (2015a). Overview of the problems resulting from biofilm contamination in the dairy industry. In K. H. Teh, S. Flint, J. Brooks, & G. Knight (Eds.), *Biofilms in the dairy industry* (pp. 49–64). Wiley-Blackwell, John Wiley & Sons, Inc.
Seale, B., Burgess, S., Flint, S., Brooks, J., Bremer, P., & Parkar, S. (2015b). Thermophilic spore-forming bacilli in the dairy industry. In K. H. Teh, S. Flint, J. Brooks, & G. Knight (Eds.), *Biofilms in the dairy industry* (pp. 112–137). Wiley-Blackwell, John Wiley & Sons, Inc.
Simões, M., Simões, L. C., & Vieira, M. J. (2010). A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology, 43*, 573–583.
St-Gelais, D., Haché, S., & Gros-Louis, M. (1992). Combined effects of temperature, acidification, and diafiltration on composition of skim milk retentate and permeate. *Journal of Dairy Science, 75*, 1167–1172.
Suwarno, S. R., Chen, X., Chong, T. H., McDougald, D., Cohen, Y., Rice, S. A., et al. (2014). Biofouling in reverse osmosis processes: The roles of flux, crossflow velocity and concentration polarization in biofilm development. *Journal of Membrane Science, 467*, 116–125.
Tang, X., Flint, S. H., Bennett, R. J., & Brooks, J. D. (2010). The efficacy of different cleaners and sanitisers in cleaning biofilms on UF membranes used in the dairy industry. *Journal of Membrane Science, 352*, 71–75.
Tang, X., Flint, S., Bennett, R., Brooks, J., & Zain, S. N. M. (2015). Biofilm contamination of ultrafiltration and reverse osmosis plant. In K. H. Teh, S. Flint, J. Brooks, & G. Knight (Eds.), *Biofilms in the dairy industry* (pp. 138–153). Wiley-Blackwell, John Wiley & Sons, Inc.
Tang, X., Flint, S. H., Brooks, J. D., & Bennett, R. J. (2009). Factors affecting the attachment of micro-organisms isolated from ultrafiltration and reverse osmosis membranes in dairy processing plants. *Journal of Applied Microbiology, 107*, 443–451.
Teh, K. H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., & Lindsay, D. (2014). Biofilm – an unrecognised source of spoilage enzymes in dairy products? *International Dairy Journal, 34*, 32–40.
Trägårdh, G. (1989). Membrane cleaning. *Desalination, 71*, 325–335.
Wood, T. L., Guha, R., Tang, L., Geitner, M., Kumar, M., & Wood, T. K. (2016). Living biofouling-resistant membranes as a model for the beneficial use of engineered biofilms. *Proceedings of the National Academy of Sciences, 113*, e2802–e2811.
Yan, S. H., Hill, C. G., & Amundson, C. H. (1979). Ultrafiltration of whole milk. *Journal of Dairy Science, 62*, 23–40.
Yeon, K. M., Cheong, W. S., Oh, H. S., Lee, W. N., Hwang, B. K., Lee, C. H., et al. (2009). Quorum sensing: A new biofouling control paradigm in a membrane bioreactor for advanced wastewater treatment. *Environmental Science and Technology, 43*, 380–385.
Yuan, L., Burmølle, M., Sadiq, F. A., Wang, N., & He, G. (2018). Interspecies variation in biofilm-forming capacity of psychrotrophic bacterial isolates from Chinese raw milk. *Food Control, 91*, 47–57.