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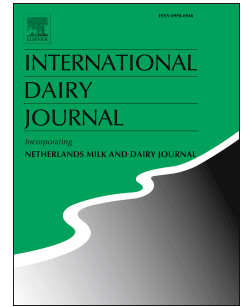


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1 **Influence of feed temperature to biofouling of ultrafiltration membrane during**
2 **skim milk processing**

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23

24

25 ABSTRACT

26

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

38

39

40

41 1. Introduction

42

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

58 The main strategy used to control biofouling in the dairy industry is to clean
59 membranes daily with a clean-in-place (CIP) system, circulating acid and alkaline
60 solutions at the membrane surface following a filtration time of up to 24 h (Anand et al.,
61 2014; Berg et al., 2014). This cleaning process restores the membrane permeation flux
62 (Trägårdh, 1989). However, traditional CIP may not remove or kill bacteria cells
63 adhered on stainless steel or filtration membrane surfaces (Bénézech & Faille, 2018;
64 İpek & Zorba, 2018; Marka & Anand, 2017), even with optimised CIP protocols
65 (Kumari & Sarkar, 2014). At the laboratory scale, innovative biofilm removal or control

66 strategies dedicated to the membrane separation industry have been suggested: the use
67 of ozonised water (Henderson et al., 2016), surfactants (Hijnen et al., 2012) or specific
68 enzymes (Khan et al., 2014; Tang, Flint, Bennett, & Brooks, 2010), quorum quenchers
69 (Yeon et al., 2009), quorum quenching bacteria (Kampouris et al., 2018; Oh et al.,
70 2017) or bioengineered biofilms (Wood et al., 2016). The use of specific enzymes is
71 actually recommended in the industry to control short-term issues related to biofouling,
72 but are still inefficient in a long-term purposes (Simões et al., 2010).

73 The complete removal of biofilms from filtration membranes may not be
74 possible (Bucs et al., 2018; Simões et al., 2010). Instead of eradicating them from
75 filtration systems, it appears more realistic to delay biofilm formation (Bucs et al.,
76 2018), or to reduce their negative impact through the selection of operational parameters
77 that limit the growth of problematic bacteria such as those that are thermoresistant.
78 Recently, the chemistry of the membrane material was found to affect the persistence of
79 a problematic spore-former (*Bacillus* sp.) on filtration membranes following whey
80 ultrafiltration (UF) (Chamberland et al., 2017a). Lowering the temperature of the feed
81 also appears a good strategy since it reduced the incidence of biofouling on reverse
82 osmosis membranes in the desalination industry (Farhat, Vrouwenvelder, Van
83 Loosdrecht, Bucs, & Staal, 2016), and the adhesion of *Streptococcus* sp. on PES
84 membranes during milk or whey UF (Chamberland, Lessard, Doyen, Labrie, & Pouliot,
85 2017b).

86 Two common feed temperatures are used during UF of dairy fluids. Although
87 higher feed temperatures (i.e., 50 °C) provide higher permeation fluxes (Méthot-Hains
88 et al., 2016; St-Gelais, Haché, & Gros-Louis, 1992; Yan, Hill, & Amundson, 1979),
89 better microbial quality of the retentate is expected when filtration is performed at a
90 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.

103

104 **2. Material and methods**

105

106 *2.1. Milk source*

107

108 A different batch lot of raw milk (temperature < 4 °C, pH between 6.65–6.75)
109 was obtained from a local dairy prior each experiment. Milk was skimmed (< 0.01%
110 fat) and pasteurised (75 °C for 16 s) as previously described (Chamberland et al.,
111 2017b). The pasteurised milk was directly collected in a sterilised 20 L feed tank (Cole-
112 Parmer, Montreal, QC, Canada) called the milk tank, and was stored at 4 °C. The time
113 between the pasteurisation and the beginning of each experiment was 12 h or less.

114

115 2.2. *Biofilm reactor assembly and operation*

116

117 A biofilm reactor (CBR 90, BioSurfaces technologies corporation, Bozeman,
118 MN, USA) that mimics the filtration environment was used to study the attachment of
119 bacteria on 10 kDa polyethersulfone (PES) UF membranes (ST membrane, Synder,
120 Vacaville, CA, USA) (Fig. 1). Membrane coupons measuring 6 cm² were fixed on eight
121 membrane holders, between the holders and the restraining frames specifically designed
122 for the biofilm reactor. The eight holders are placed in such a way in the bioreactor that
123 the flow condition is identical for each membrane (Fig. 1B). The milk tank was always
124 maintained at 4 °C in an incubator (MIR-153, Sanyo, Osaka, Japan) throughout the
125 biofilm development experiments (Fig. 1). A peristaltic pump (Masterflex, Model
126 7518-00, Barnant Company, Barrington, IL, USA) continuously transferred milk from
127 the milk tank to the biofilm reactor through silicone tubing (Tubing M-Flex L/S 16,
128 Cole-Parmer, Montreal, Canada) at a constant flow rate of 0.25 mL s⁻¹ (flow rate value
129 between those of Bremer, Fillery, and McQuillan (2006) and Dufour, Simmonds, and
130 Bremer (2004)). The stirring rate inside the reactor was 180 rpm, as described by Tang
131 et al. (2009), to generate a flow regime considered as turbulent according to
132 Buckingham-Meyer, Goeres, and Hamilton (2007).

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

138 Efforts were made to mimic as much as possible the filtration system
139 environment by operating the biofilm reactor in the continuous mode, at representative

140 operating temperatures, with the highest level of turbulence possible, and with
141 pasteurised milk having its natural microflora (thermo-resistant bacteria and
142 environmental contaminants). However, the turbulent flow regime and shear stress at
143 membrane's surface in a filtration system could not be reproduced using the bioreactor.

144

145 2.3. *Bioreactor preparation*

146

147 Prior to each experiment, the biofilm reactor and its different parts (tanks and
148 tubing) were autoclaved at 121 °C for 15 min. Membrane coupons were then fixed
149 aseptically on the membrane holders and a conventional CIP of the system
150 (conditioning step) was performed as recommended by the membrane manufacturer.
151 Briefly, the CIP procedure consisted of alkaline (pH 10.5), acid (pH 2.0) and
152 chlorinated alkaline (pH 10.5, 150 ppm of free chlorine) cleaning steps, executed
153 consecutively. Cleaning solutions were prepared with commercial chemicals:
154 Membra-base 210 (Sani-Marc, Victoriaville, QC, Canada), Ultrasil 75 (Ecolab, Saint
155 Paul, MN, USA) and Chloreco (Sani-Marc). Each cleaning step was performed for
156 30 min at 50 °C. A rinsing step with deionised water at 50 °C preceded and followed
157 each individual step of the CIP. The stirring rate of 180 rpm was maintained in the
158 biofilm reactor during the cleaning procedure. A cleaning step under the same
159 conditions was also performed at the end of each experiment.

160

161 2.4. *Membrane and milk sampling*

162

163 During experiments performed at 15 °C, membranes were collected with a
164 sterile scalpel after the membrane conditioning step (0 h), after 20 h, 24 h, 28 h, 32 h,

165 36 h, 48 h and after the final cleaning step at the end of the experiment. Considering the
166 shorter development time of bacteria at warmer temperatures, the membranes were
167 collected in shorter intervals of time at 50 °C: after the membrane conditioning step,
168 after 7.5 h, 10 h, 12.5 h, 15 h, 20 h and after the last cleaning step. Prior to being stored
169 at –80 °C until DNA extraction, membranes were gently rinsed in a sterile phosphate
170 buffered saline (PBS) solution (pH 7.4) to remove planktonic bacteria from membranes
171 (Anand, Hassan, & Avadhanula, 2012).

172 Milk inside the biofilm reactor was sampled at the same time points as the
173 membranes. For each sampling time, three samples of 2 mL were pelleted and stored at
174 –80 °C until DNA extraction.

175

176 2.5. Targeted genomic analysis of the bioreactor microbiome

177

178 2.5.1. Genomic DNA extraction

179 For both temperature conditions, genomic DNA, which contains DNA from
180 dead and viable cells, was extracted in duplicate from the milk and the membrane
181 samples as described previously (Chamberland, Lessard, Doyen, Labrie, & Pouliot,
182 2017c), except that sodium acetate (pH 5.2) at a final concentration of 0.3 M was used to
183 precipitate the DNA (Sambrook & Russell, 2001).

184

185 2.5.2. High-throughput sequencing and bioinformatics

186 Amplicon sequencing targeting the V6-V8 region of the 16S ribosomal RNA
187 (rRNA) gene was performed on a Miseq sequencer at the Institut de Biologie Intégrative
188 et des Systèmes (Université Laval, Québec, QC, Canada). Two replicates for each
189 temperature condition were used for the sequencing step. Raw demultiplexed paired-end

190 reads of each replicate were deposited in the GenBank database under the accession
191 number SRP140914 (first replicate, 3,285,298 raw demultiplexed reads) and
192 SRP150623 (second replicate, 3,169,154 raw demultiplexed reads). The computations
193 of the reads of each replicate were processed separately, as previously described
194 (Chamberland et al., 2017a), with the modifications described next. Computations were
195 done with the pipeline FROGS from the Galaxy portal of the INRA MIGALE
196 Bioinformatics platform (Jouy-en-Josas Cedex, France; Escudié et al., 2017; Goecks et
197 al., 2010). Following reads preprocessing (quality filter, read trimming and read
198 assembly), contiged reads were clustered with the Swarm clustering method using the
199 denoising clustering step and the suggested aggregation distance of 3 (Mahé, Rognes,
200 Quince, de Vargas, & Dunthorn, 2014). A first chimera filter was performed with
201 VSEARCH (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Operational taxonomic
202 units (OTU) with abundance lower than 0.005% were removed (Bokulich et al., 2013).
203 The SILVA database (release 132, December 13, 2017) was used to perform the OTU
204 affiliations. Each OTU was manually inspected a second time to remove undetected
205 chimeras. The final OTU tables obtained for each replicate were finally merged by
206 taking the sum of the absolute abundance of each taxon. Ecological metrics (alpha- and
207 beta-diversity) were computed with the Mothur pipeline (v1.35.1) (Schloss et al., 2009),
208 as previously described (Chamberland et al., 2017c).

209

210 2.5.3. *Quantification of bacterial growth*

211 The number of 16S rRNA gene copies found on membranes and in milk at each
212 time point were quantified by qPCR as described previously (Chamberland et al.,
213 2017a).

214

215 2.6. *Statistical analysis*

216

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

223

224 3. **Results**

225

226 3.1. *Quantification of the number of 16S rRNA gene copies on membranes*

227

228 Changes in bacterial communities in milk and on membranes were studied as
229 function of time for two temperatures, 15 °C and 50 °C (Fig. 2). The bacterial
230 communities observed on membranes were significantly different depending on whether
231 the membranes were used at 15 °C or 50 °C (Fig. 2A). At 50 °C, a significant increase
232 in the number of 16S rRNA gene copies was observed throughout the experiment
233 ($P < 0.05$, Fig. 2A). The number of gene copies per cm^2 increased significantly from
234 $3.21 \pm 0.12 \log_{10}$ after the first cleaning to $5.40 \pm 0.97 \log_{10}$ gene copies per cm^2
235 ($P < 0.05$) after 7.5 h, finally reaching a maximum of $8.83 \pm 1.58 \log_{10}$ gene copies per
236 cm^2 after 15 h ($P < 0.05$, Fig. 2.A). Following the cleaning step performed after 20 h of
237 operation, the number of gene copies ($5.57 \pm 3.38 \log_{10}$ gene copies per cm^2) was still
238 significantly higher than at the beginning of the process ($3.21 \pm 0.12 \log_{10}$ gene copies
239 per cm^2 , $P < 0.05$, Fig. 2.A).

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

245

246 3.2. Composition of bacterial communities formed on membranes

247

248 The temperature of the feed also affected the diversity and richness of bacteria
249 observed on membranes. Globally, communities formed at 15 °C had more diversity
250 and richness (**Error! Reference source not found.**). For example, the diversity index
251 (Inverse Simpson) was between 1.00 and 6.57 on membranes operated at 50 °C while it
252 was between 8.35 and 20.58 at 15 °C (Table 1). In the same vein, membranes operated
253 at 50 °C had a lower richness, estimated between 7.84 to 67.83 species, compared with
254 between 63.51 and 180.70 at 15 °C (Chao index, Table 1).

255 Communities formed at 50 °C were dominated by the Bacilli class (more than
256 60.15% from 7.5 h, Supplementary material Table S1). In contrast, those formed at the
257 colder temperature were composed of Actinobacteria (13.46% to 26.26%), Bacteroidia
258 (12.53% to 27.00%), Bacilli (8.01% to 20.69%), α - and γ -Proteobacteria (2.77% to
259 23.94% and 21.80% to 35.27%, respectively) classes (Supplementary material Table
260 S2). Low proportions of the Clostridia class were also observed (0.04 to 4.32%) at
261 15 °C (Supplementary material Table S2), while this class was not present at 50 °C, or
262 in trace concentrations (0.67% after 7.5 h, Supplementary material Table S1).

263 Of the bacterial genera observed on membranes, the *Lactococcus* genus was the
264 most abundant during the first part of the experiment performed at 50 °C, representing

265 37.18% of the community on the membrane sampled after 7.5 h (Fig. 2A). However, at
266 this temperature, exponential growth of *Bacillus* spp. occurred, presumably primarily
267 *B. licheniformis* based on a local BLAST alignment, from 10 h to 15 h of operation. The
268 number of 16S gene copies affiliated to the *Bacillus* genus increased from 1.66 to
269 $6.14 \log_{10}$ gene copies per cm^2 between these sampling times (Fig. 2A). The *Bacillus*
270 genus reached stationary phase at 15 h of operation and persisted on membranes, even
271 after cleaning.

272 At 15 °C, psychrotrophic (cold-tolerant) bacterial genera were observed on
273 membranes, such as *Corynebacterium* (ratios between 3.51 to 12.28%), *Halomonas*
274 (ratios between 1.10% and 7.67%), *Pseudomonas* (ratios between 0.09 to 11.65%) or
275 *Psychrobacter* (ratios between 10.26% to 26.75%) (Fig. 2A and Table S2).
276 *Psychrobacter* was the most abundant genus, however, no one genus was dominant, and
277 no exponential growth was observed throughout the 48 h-experiments (Fig. 2A). The
278 *Bacillus* genus was only found in low ratios (0.19% to 3.92%) at this temperature (Fig.
279 2A).

280

281 3.3. Changes in bacterial diversity in milk inside the bioreactor

282

283 As on the membranes, there were few variations in the proportions of bacterial
284 OTU in milk circulating in the bioreactor at 15 °C. A decrease in the number of 16S
285 rRNA gene copies was suspected, from $6.08 \pm 1.41 \log_{10}$ gene copies per mL at the
286 beginning of the experiment to $4.52 \pm 0.12 \log_{10}$ gene copies per mL after 48 h of
287 operation, but the difference was not significant ($P > 0.05$, Fig. 2B). At 50 °C, an
288 increase in *Bacillus* spp. was observed in milk, similar to the finding with the
289 membranes, but an increase in the number of 16S gene copies was only significant after

290 15 h of operation, where the number of copies reached $8.49 \pm 0.67 \log_{10}$ gene copies
291 per mL ($P < 0.05$, Fig. 2B).

292

293 4. Discussion

294

295 4.1. Operating at 50 °C, a race against spore-former bacteria

296

297 The most important predictable issues resulting from UF of dairy fluid at 50 °C
298 is the formation of biofilms composed of thermophilic bacteria on UF membranes
299 during the process (Anand et al., 2014; Burgess, Lindsay, & Flint, 2010; Pompei et al.,
300 1973), and the subsequent contamination of the feed, as seen in milk circulating inside
301 the bioreactor (Fig. 2B). These bacteria, including the commonly found
302 *Anoxybacillus flavithermus*, *Geobacillus* spp. and *Bacillus* spp. are generally not
303 pathogenic, but their biofilm-forming and spore-forming abilities make them extremely
304 difficult to remove or kill using conventional heat treatments, and their heat-stable
305 enzymes may also affect the quality of dairy products (Burgess et al., 2010; Cho et al.,
306 2018; Sadiq et al., 2017). Complete CIP may be sufficient to kill them on stainless steel,
307 which support high temperature cleaning (Parkar, Flint, & Brooks, 2004). However, on
308 weaker materials such as polymeric filtration membranes, it was observed that biofilms
309 of *Bacillus* spp. are one of the most resistant to CIP solutions (Anand & Singh, 2013).

310 This study determined that a 15 h run at 50 °C (time needed to reach a stationary
311 phase on membranes) allows mature and saturated biofilm composed of spore-former
312 bacteria (*Bacillus* spp.) to form on a cleaned membrane during its first use. The *Bacillus*
313 genus entered exponential growth phase after approximately 10 h and grew significantly
314 in a short period of time, as found in other studies with doubling times corresponding to

315 0.25 h and to 0.52 h at this temperature (Burgess et al., 2010; Dufour et al., 2004;
316 Gauvry et al., 2017). In comparison, *Streptococcus thermophilus* biofilms may be
317 formed in 6 h in the regeneration section of a pasteuriser, at a temperature between
318 30 °C and 40 °C (Bouman, Lund, Driessen, & Schmidt, 1982; Knight, Nicol, &
319 McMeekin, 2004), while the contamination of thermophilic spore-forming bacteria
320 appears from 9 h during an evaporation process (Murphy, Lynch, & Kelly, 1999; Scott,
321 Brooks, Rakonjac, Walker, & Flint, 2007).

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

335

336 4.2. *Cold temperature as a key parameter to delay biofilm formation*

337

338 As mentioned previously, there is a trend in the dairy industry to perform
339 continuous unit operations such as UF at colder temperatures (Tang, Flint, Brooks, &

340 Bennett, 2009) to provide dairy products with a better microbial quality, even if this
341 reduces membrane performance because of higher milk viscosity (Kapsimalis & Zall,
342 1981; Yan et al., 1979).

343 As mentioned by Yuan, Burmølle, Sadiq, Wang, and He (2018), few studies
344 have looked at biofilm formation assays at cold temperatures and whether some
345 psychrotrophic bacteria found in raw milk, such as *Pseudomonas fluorescens*, have
346 increased biofilm-forming abilities at colder temperatures (Aswathanarayan & Vittal,
347 2014). Indeed, biofilms have already been found on dairy industrial filtration
348 membranes operated around 15 °C with pasteurised milk (Chamberland et al., 2017c;
349 Tang et al., 2009).

350 In this study, membranes operated at cold temperature were colonised with
351 bacteria such as *Pseudomonas*, *Lactococcus* and *Sediminibacterium*, as observed
352 previously following 5 h UF at 10 °C in a model cross-flow filtration system
353 (Chamberland et al., 2017b). However, this 48 h experiment did not provide sufficient
354 evidence for biofilm formation or for growth on the membranes since the numbers of
355 16S rRNA gene copies on membranes did not differ significantly across the times
356 sampled ($P > 0.05$, Fig. 2A). At 15 °C, both psychrotrophic and mesophilic bacteria can
357 grow in the dairy environment (Seale et al., 2015a). However, mesophilic bacteria such
358 as lactic acid bacteria have a slow growth rate at this temperature (Adamberg, Kask,
359 Laht, & Paalme, 2003) and psychrotrophic bacteria from raw milk that survive
360 pasteurisation are scarce (Coghill, 1982; Quigley et al., 2013a). Even if it was
361 demonstrated that a psychrotrophic genus such as *Pseudomonas* was more resistant to
362 heat treatment than previously stated (Quigley et al., 2013a), most of the psychrotrophic
363 bacteria found in pasteurised milk originate through post-contamination from the water
364 source or the plant environment (Chamberland et al., 2017b; Coghill, 1982; Quigley et

365 al., 2013b).

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

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

386

387 4.3. *The representativeness of the biofilm reactor*

388

389 Necessarily, results obtained from a biofilm reactor in this study did not reflect

390 perfectly the reality of industrial filtration systems. Indeed, according to observations
391 made in the desalination industry (reverse osmosis membranes), a higher flow velocity
392 in filtration system or the presence of a concentration polarisation at the membrane
393 surface may affect the time needed to form biofilms on membranes (Suwarno et al.,
394 2014). On the other hand, the use of a biofilm reactor helped to generate membrane
395 samples at different time points without affecting the retentate flow, and to determine
396 more precisely the impact of the temperature in a controlled environment. In
397 comparison with other studies made previously, the ratio of the bacteria among
398 communities forming on membranes in model filtration system or in the biofilm reactor
399 were different (Chamberland et al., 2017a,b). However, common dairy bacterial genus
400 such as *Bacillus* spp., *Lactococcus* spp., *Pseudomonas* spp. or *Streptococcus* spp. were
401 observed in both systems (Chamberland et al., 2017a,b).

402

403 **5. Conclusions**

404

405 The temperature of the feed, as shown in this study, is a crucial parameter to
406 consider for preventing biofilm formation on membranes. Indeed, at the industrial scale,
407 the conclusions presented here cannot be taken as an argument to perform cold milk UF
408 over 48 h. However, this study revealed that in a system wherein bacterial contaminants
409 are well controlled (system perfectly cleaned and cleaned with good quality water), the
410 biofilm formation rate on membranes is considerably slower at 15 °C than at 50 °C.

411 Indeed, in a system where the daily cleaning procedures are rigorously performed,
412 biofouling may not be an issue at 15 °C. However, as noted, additional work is needed
413 to determine how psychrotrophic biofilms persist on filtration membranes and affect
414 cleaning efficiency in real filtration systems. Industrially, if the feed viscosity does not

415 permit operation at cold temperatures, or if industrial installations are not available,
416 filtration at 50 °C may be mandatory. Then, the operating time should be limited to 10 h
417 to avoid the exponential growth of thermoresistant bacteria such as *Bacillus* sp. on
418 membranes.

419

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421

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431

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1 **Figure legends**

2

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

8

9 **Fig. 2.** Portrait of bacterial communities formed on membranes (A) and bacteria found
10 in milk (B) inside the bioreactor at 15 °C or 50 °C. Bars with the same letter do not have
11 a significantly different gene copy number (Fischer's least significant difference test, P
12 > 0.05 , $n = 3$). The percentages indicate the proportion of each genus.

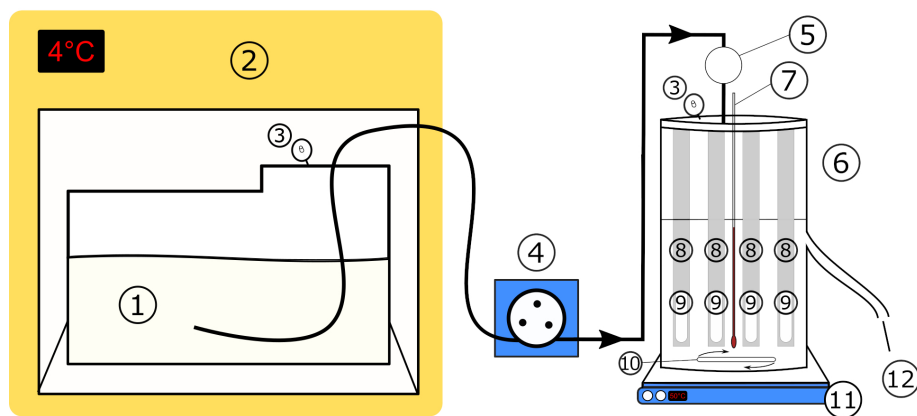
13

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.

A**B**