Efficacy of Organic Peroxyacids for Eliminating Biofilm Preformed by Microorganisms Isolated from Dairy Processing Plants

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ABSTRACT The aim of this study was to evaluate the ability of microorganisms isolated from the dairy industry to form biofilms and to investigate the efficacy of organic peroxyacids (peracetic, perpropionic, and perlactic acids and BioDestroy) to eradicate those biofilms. Eighteen microorganisms were isolated from Quebec dairy processing plants that have issues associated with biofilm formation and were presumptively identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry. The single-species biofilm-producing ability of the isolates was then evaluated using 96-well microplates. Eight out of 18 of these isolates were identified as moderate or strong biofilm producers, and 10 out of 18 were negative or weak biofilm producers. The efficacy of the above-mentioned disinfectants was tested on the stronger biofilm-producing bacteria using the MBEC (minimum biofilm eradication concentration) assay. After 5 min, all disinfectants tested successfully eradicated both the single and mixed biofilms when applied following the recommended concentration. However, the efficacy of organic peroxyacids was significantly variable at lower concentrations. For example, 25 ppm of BioDestroy was sufficient to eradicate all the biofilms, except for *Pseudomonas azotoformans* PFI1A. Unfortunately, microscopic observations highlighted those dead cells were still attached to the surfaces. In conclusion, our results suggest that some microorganisms found in dairy plants can produce tenacious biofilms that are still susceptible to disinfectants, including organic peroxyacids. Further studies would be needed to confirm these observations using a dynamic method to mimic *in vivo* conditions.

IMPORTANCE Biofilm-forming microorganisms are a major issue in the food industry, including the dairy industry, because of their negative impact on product quality. Biofilms are difficult to remove by clean-in-place (CIP) procedures commonly used in processing plants and may be less sensitive to sanitizers. Therefore, it is important to identify these microorganisms to develop biofilm control strategies. The results gathered in the present study could contribute to this aim, even though it was carried out using only static methods.

KEYWORDS dairy industry, processing plant, biofilm eradication, organic peroxyacid

Despite compliance with hygiene standards throughout the dairy industry, the risk of producing noncompliant or atypical dairy products remains ever present (1, 2). In Canada, an estimated 21% of dairy products are not distributed due to contamination by spoilage or pathogenic microorganisms (3). Spoilage microorganisms can reduce the value of dairy products and derivatives by producing gas, acid, or extracellular enzymes (1, 2). For example, *Pseudomonas fluorescens* and *Pseudomonas azotoformans* are associated with milk discoloration (4, 5). Pathogenic microorganisms can cause serious illnesses (1, 2). For example, *Listeria monocytogenes* has been identified as a cause of foodborne disease outbreaks with a high mortality rate, especially for immunocompromised individuals (6, 7).

Some of these microorganisms are particularly problematic because of their ability to form persistent biofilms on a wide variety of inert surfaces, including stainless steel food processing equipment (8). Studies have also reported that pathogenic bacteria, including *L. monocytogenes*, can associate with preexisting multispecies biofilms, thereby increasing their resistance to sanitizers (9). Bacteria can easily detach from biofilms and contaminate the food products that come in contact with the surface. This often forces product recalls, causing economic losses. In the case of an undetected pathogen contamination, consumption of the products can cause the spread of foodborne illnesses. In addition to these problems, biofilms can impede heat transfer and cause mechanical blockage of process flow (10), accelerate corrosion, and force premature replacement of equipment (11). Dairy biofilms are complex microbial ecosystems that are difficult to eradicate because of their resistance to common industrial disinfectants (8, 12). Better knowledge of dairy processing plant microflora and its ability to produce biofilms is needed to effectively address this problem. Most of the research in this field focuses on pathogens, such as *L. monocytogenes* and the most common spoilage microorganisms, such as *P. fluorescens* (8, 13), but knowledge on dairy mixed biofilms is too limited. Because of their decreased sensitivity to biocides, complex biofilms are an important issue that needs in-depth studying (14, 15).

The dairy industry generally relies on clean-in-place (CIP) procedures to control biofilms (8). This typically involves a series of washing steps, including sequential use of sodium hydroxide (caustic), nitric acid, and, in some cases, sanitizers, such as hypochlorous and peracetic acids (8, 16). Although hypochlorous acid is highly effective against bacteria, it also reacts with natural organic matter, producing toxic by-products (17, 18). Peracetic acid is a strong oxidizer and an effective broad-spectrum disinfectant that does not generate harmful by-products (15, 18). However, its effectiveness against biofilms in the food industry is highly variable depending on biofilm composition (15, 19). In recent years, the antimicrobial activity of perpropionic, perlactic, and other peroxyacids has been studied, and they appear to be a promising avenue (18). In fact, the presence of an additional methyl group makes perpropionic acid more stable than peracetic acid, which can be explosive (20).

To our knowledge, the biofilm-producing ability of specific spoilage microbial species found in dairy processing plants in Quebec and their sensitivity to organic peroxyacids other than peracetic acid alone has not received any focused attention. Therefore, the purpose of the present study was to evaluate biofilm formation by spoilage microorganisms isolated in Quebec dairy processing plants and then perform a preliminary screening of several organic peroxyacids as antibiofilm agents by observing their effectiveness at eliminating single and mixed biofilms formed by these microorganisms. Although this study was conducted using only static methods, the results could contribute to the development of new biofilm control strategies.

RESULTS AND DISCUSSION

Despite the strict hygiene requirements imposed on the dairy industry, milk can still contain spoilage or pathogenic microorganisms (2, 8, 21). Biofilms produced by these microorganisms can adhere to most materials, including stainless steel, which most dairy processing equipment is made of, increasing the risk of microbial contamination (8). Dairy biofilms are complex microbial ecosystems comprising water, proteins, minerals, and polysaccharides, inhabited by spoilage and sometimes pathogen microorganisms (8, 12). They make for an unwanted built-in single- or mixed-species microbial inoculum, thereby acting as an ever-present potential threat to dairy product quality (1, 2). All equipment surfaces must be cleaned and disinfected regularly to suppress biofilm formation. Unfortunately, biofilms remain difficult to eradicate, since microorganisms encased in them are less sensitive to disinfectants (8, 12). Scientific studies on the characterization of microorganisms isolated from industrial food-processing settings in Quebec and their sensitivity to organic peroxyacids other than peracetic acid are lacking. The present study focuses on performing a preliminary screening of the biofilm-forming ability of microorganisms found in Quebec dairy processing plants

TABLE 1 Microbial isolates used in this study[a]

Isolate	Presumptive identity	Origin	Associated characteristic	Culture conditions	Biofilm absorbance[b] (A_{570})
PFl1A	*Pseudomonas azotoformans*	Plate exchanger	Alteration of milk appearance (blue/grey staining)	TSB, 16 h, 30°C	1.58
PFr1A	*Pseudomonas fragi*	Plate exchanger	Alteration of milk texture (filamentous)	TSB, 48 h, 30°C	0.22
B1	*Candida parapsilosis*	Pump	RC	TSB, 16 h, 30°C	0.17
B11	*C. parapsilosis*	Pump	RC	TSB, 16 h, 30°C	0.12
B12	*C. parapsilosis*	Pump	RC	TSB, 16 h, 30°C	0.11
B27	*Microbacterium arborescens*	Freezer	RC	TSB, 16 h, 30°C	0.09
B29A	*Brevundimonas vesicularis*	Freezer	RC	TSB, 48 h, 30 C	2.31
B30	*B. vesicularis*	Freezer	RC	TSB, 16 h, 30°C	0.63
B33	*B. vesicularis*	Freezer	RC	TSB, 16 h, 30°C	0.30
B36	*B. vesicularis*	Freezer	RC	TSB, 16 h, 30°C	3.62
B37	*B. vesicularis*	Freezer	RC	TSB, 48 h, 30°C	1.13
B38	*B. vesicularis*	Freezer	RC	TSB, 16 h, 30°C	1.24
B40	*B. vesicularis*	Freezer	RC	TSB, 16 h, 30°C	3.54
Pa10	*Paenibacillus odorifer*	Milk	HBCC	TSB, 16 h, 30°C	0.04
PG	*Microbacterium lacticum*	NS	NS	TSB, 16 h, 30°C	0.23
S18864	*Serratia liquefaciens*	NS	NS	TSB, 16 h, 30°C	0.88
K4	*Kocuria rhizophila*	NS	NS	TSB, 16 h, 30°C	0.18
Pa18725	*Paenibacillus polymixa*	Milk	HBCC	TSB, 16 h, 30°C	0.02
Lm 1045[c]	*Listeria monocytogenes*	Raw milk cheese handwork	2008 outbreak in Québec, Canada (50)	TSB, 16 h, 30°C	
Lm 1046[d]	*L. monocytogenes*	Pasteurized milk cheese	NS	TSB, 16 h, 30°C	
Positive control (C+)	*Pseudomonas fluorescens* 13525	ATCC	Not applicable	TSB, 16 h, 30°C	0.69

[a]RC, repeated contaminations; HBCC, high bacterial cell count; NS, not specified.
[b]The ability of isolates to form biofilm was classified as strong ($A_{570} \geq 1.5$), moderate ($0.5 \geq A_{570} < 1.5$), or weak or negative ($A_{570} \leq 0.5$).
[c]Serotype 1/2a and pulsotype 93.
[d]Biochemically *L. monocytogenes*, serologically untypeable.

and evaluating the sensitivity to organic peroxyacids of single- and mixed-species biofilms formed by these bacteria.

Preliminary identification of microorganisms isolated in dairy processing plants in Quebec. Eighteen microbial isolates were isolated from dairy processing plants in Quebec that have experienced problems with biofilm formation. Biofilm formation on equipment surfaces is automatically suspected if contamination of the dairy products persists even after an additional cleaning cycle. Based on matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis, 15 microbial isolates were presumptively identified as bacteria (Table 1). Microorganisms frequently found in dairy biofilms worldwide include *Pseudomonas* spp., *Aeromonas* spp., *Staphylococcus* spp., *Bacillus* spp., lactic acid bacteria, *Enterobacteriaceae*, and *Listeria* spp. (8). They tend to be heat-sensitive bacteria and are often found in milk pipelines or bulk tanks prior to pasteurization, while thermophiles are found frequently in heating equipment (8). The results gathered show that *Pseudomonas* spp. were often present in Quebec processing plants, but *B. vesicularis* was the most frequently isolated organism from dairy biofilms. Indeed, among the 15 microbial isolates, 7 were presumptively identified as *B. vesicularis* (Table 1). *Brevundimonas* spp., including *B. vesicularis*, are Gram-negative bacteria often found in clinical specimens and human samples (eyes, urine, central nervous system, and lungs) and are considered emerging global opportunistic pathogens (22–24). No information about *Brevundimonas vesicularis* and its tolerance to cold was found in the literature. However, isolates affiliated with the genus *Brevundimonas* were isolated from extremely cold environments (25). Furthermore, other species of *Brevundimonas* were identified as psychrotolerant (26–28). These findings could explain why the isolates of *Brevundimonas vesicularis* survive in the freezer.

**TABLE 2** MBEC determined for each isolate and each disinfectant

Disinfectant	MBEC (ppm)						
	B40 (S)	B40 (M)	1045	1046 (S)	1046 (M)	S18864	PFl1A
Hydrogen peroxide	100,000	50,000	100,000	100,000	100,000	100,000	100,000
Perlactic acid	100	50	100	100	100	100	100
Perpropionic acid	500	25	25	25	25	25	500
Peracetic acid	500	50	25	25	25	25	500
BioDestroy	25	25	25	25	25	25	500

Among the 18 microbial isolates, some were responsible for altering the appearance or texture of milk (*Pseudomonas* spp.), while some were responsible for repeated contaminations despite multiple cleaning cycles (*Candida parapsilosis*, *B. vesicularis*, and *Microbacterium arborescens*) (Table 1). All the isolates were sampled from various locations in the dairy plant, including the plate exchanger, the pump, the freezer, and the final food product itself (Table 1). Some were found on more than one piece of equipment, but each microorganism was usually associated with a particular location, such as the plate heat exchanger (*Pseudomonas* spp.) or the pump (*Candida parapsilosis*) (Table 1). These findings are consistent with other studies worldwide that state that biofilms grow mostly on surfaces that are difficult to clean, for example, milk pipelines or ultrafiltration membranes, and on rough surfaces, including stainless steel (8, 29–32).

Evaluation of the ability of microorganisms isolated in dairy processing plants in Quebec to form biofilms. The single-species biofilm-producing ability of 18 isolates was evaluated using 96-well microplates, a static method commonly used for biofilm assessment. This is an inexpensive and easy-to-use method allowing a high-throughput screening of the adhered cells. The results show a wide range of biofilm-forming ability among species and isolates (Table 1). Eight of the isolates were shown to be moderate ($0.5 \geq A_{570} < 1.5$) or strong biofilm producers ($A_{570} \geq 1.5$), and 10 were shown to be weak or nonproducers ($A_{570} \leq 0.5$) (Table 1). All but one of the 7 *B. vesicularis* isolates were identified as moderate or strong biofilm producers. It has been shown in previous studies that *B. vesicularis* produces slime (polysaccharides) in liquid culture (33). In the present study, the isolates of *P. azotoformans* PFl1A and *S. liquefaciens* S18865 were also identified as moderate or strong biofilm producers (Table 1). This is consistent with other studies of biofilm production by these species in dairy settings (34–37). On the other hand, *Microbacterium* spp. and *Candida parapsilosis* were identified as weak or nonproducers despite various medium or incubation conditions being tested (data not shown).

The effectiveness of organic peroxyacid and hydrogen peroxide for eliminating biofilms. Isolates representing moderate and strong biofilm producers from three species, *Pseudomonas azotoformans* PFl1A, *Serratia liquefaciens* S18864, and *B. vesicularis* B40, were evaluated further. Two isolates of *Listeria monocytogenes* were also tested to investigate pathogenic bacterial introduction and persistence in dairy biofilms. After the selection of these 5 isolates, the efficacy of perlactic acid, perpropionic acid, peracetic acid, BioDestroy, and hydrogen peroxide was evaluated to disrupt single-species and dual-species biofilms formed on MBEC assay inoculator pegs (Table 2) using the MBEC assay, a high-throughput screening standard test method (ASTM E2799–12). The biofilm population is recorded as $\log_{10}$ CFU per unit of surface area. The effectiveness of the disinfectants is reported as the $\log_{10}$ reduction of viable cells. The MBEC assay also allows us to identify the minimal concentration of a disinfectant to eradicate a biofilm, or MBEC.

At the contact time and concentration of disinfectants usually used in dairy plants, none of the biofilms contained detectable viable cells (Fig. 1). However, interactions between disinfectants and each isolate were observed for each disinfectant over the range of concentrations tested ($P < 0.0001$ by two-way analysis of variance [ANOVA]) (Fig. 1). The peroxyacids were significantly more effective than hydrogen peroxide at the same concentrations (10,000 and 25,000 ppm) against all biofilms ($P < 0.0001$) (Fig. 1).
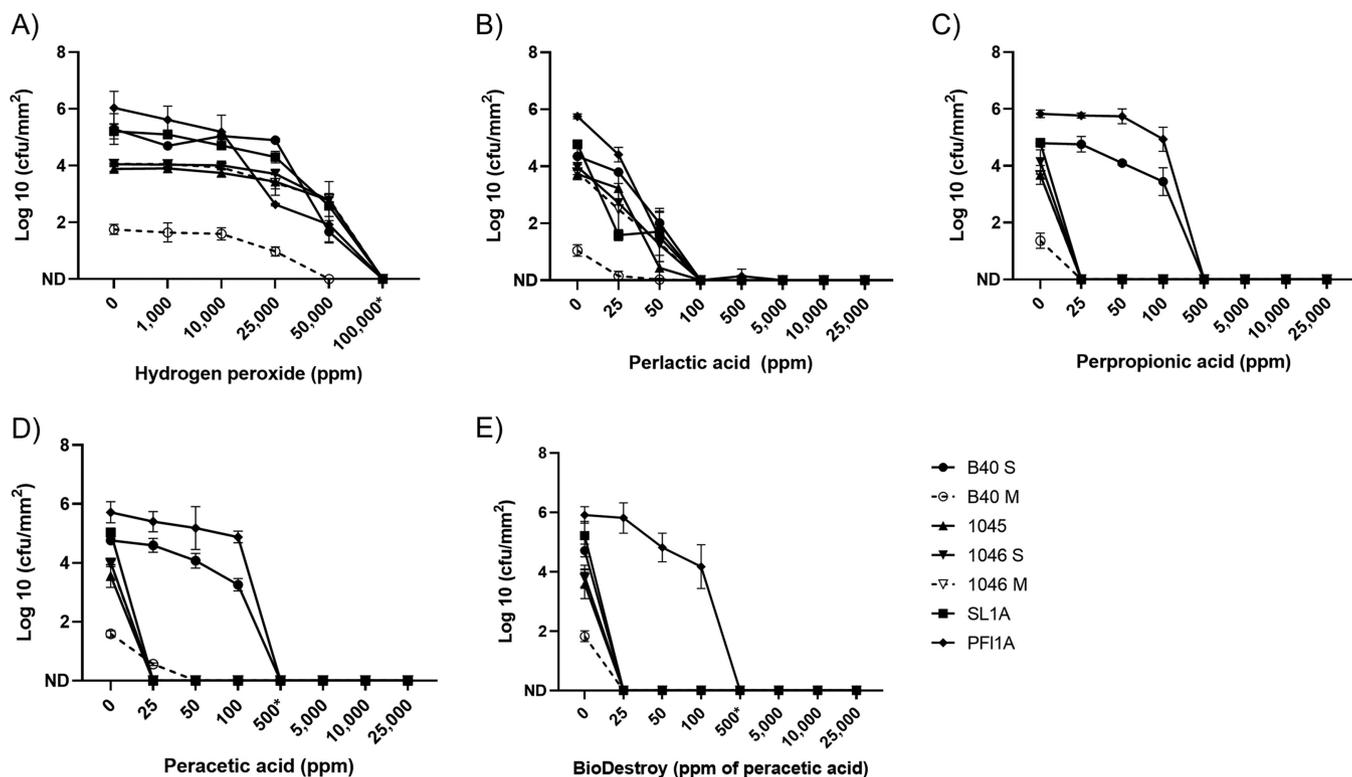
FIG 1 Viable cells in biofilms after treatment with hydrogen peroxide (A), perlactic acid (B), perpropionic acid (C), peracetic acid (D), or BioDestroy (E). Each point represents the mean from triplicate counts on three independent days for each isolate. Error bars are standard deviations (SD). The letters S and M indicate single and mixed culture, respectively. Mixed culture is composed of the isolates B40 and 1046. The abbreviation ND indicates that no colony was detected. The asterisk indicates the recommended concentrations of disinfectant by the manufacturer.

Furthermore, the MBECs were between 25 to 500 ppm and 50,000 to 100,000 ppm, respectively (Fig. 1 and Table 2). This is consistent with a biofilm study in which peracetic acid was 100% effective against *Pseudomonas aeruginosa* ATCC 15442 at 900 ppm, whereas peroxide failed to kill it at concentrations lower than 2,000 ppm (38).

A significant difference was also observed among the peroxacids (Fig. 1 and Table 2). For example, 50 to 100 ppm of perlactic acid and 25 to 500 ppm of peracetic and perpropionic acids were sufficient to eradicate all the biofilms (Fig. 1 and Table 2). These results are consistent with those of a study of their efficacy against norovirus on food contact surfaces (18). The research also shows that peracetic and perpropionic acids were the most effective at reducing viral titer by at least 3.0 $\log_{10}$, with a 5-min contact at 50 mg liter^{-1} (18). Our study highlighted that perlactic acid was the most effective against *B. vesicularis* single-species biofilms (B40S) compared to perpropionic and peracetic acids at all the tested concentrations ($P < 0.0001$). In fact, 100 ppm of perlactic acid was sufficient to eradicate B40 biofilms, while 500 ppm of perpropionic and peracetic acids was (Fig. 1 and Table 2). Interactions between concentrations and disinfectants for each isolate ($P < 0.0001$ by two-way ANOVA) (Fig. 1) were also observed. The least resistant biofilm was formed by *S. liquefaciens* S18864, which was eradicated at a concentration of 25 ppm peracetic and perpropionic acids, whereas *P. azotoformans* PFI1A biofilm resisted these peroxacids up to a concentration of 500 ppm (Fig. 1 and Table 2). Other studies have shown that peracetic acid could be of limited effectiveness against biofilms in the food industry, depending on bacterial species (15, 19). Although the polymer matrix of the biofilm is always composed of exopolysaccharides, protein, and nucleic acids, their distribution depends on the species (39). For example, the most common polysaccharide in *Staphylococcus aureus* biofilm is the poly-β(1–6)-*N*-acetylglucosamine, or PNAGn (40). The composition and structure of the biofilms formed by our isolates could be investigated further using confocal microscopy with fluorescent stains such as BOBO-3 or SYPRO Ruby or enzymatic treatments (41).
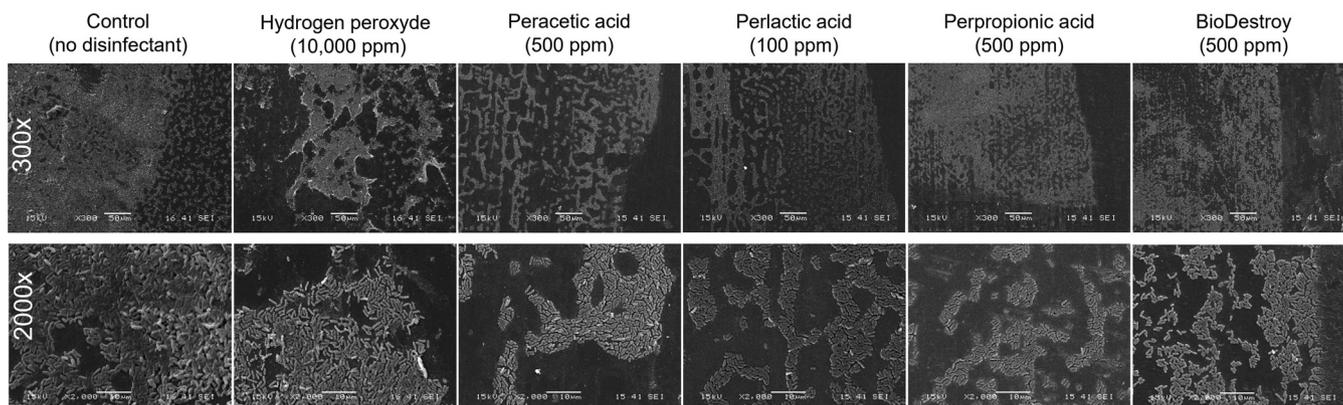
FIG 2 Scanning electron micrographs (300× and 2,000×) of *Pseudomonas azotoformans* PFl1A biofilm formed on MBEC assay pegs before (control) and after treatment with hydrogen peroxide, peracetic acid, perlactic acid, perpropionic acid, and BioDestroy at their minimum biofilm eradication concentration, or MBEC.

In addition to the organic peroxyacids, the efficacy of BioDestroy, a commercialized antibiofilm product containing peracetic acid and hydrogen peroxide, was also tested. BioDestroy was more effective at eradicating the biofilms than peracetic acid alone (Table 2). In fact, 25 ppm of BioDestroy was sufficient to eradicate the biofilms, except for *P. azotoformans* PFL1A biofilms (500 ppm) (Fig. 1 and Table 2). This could be due to the presence of an anionic surfactant or a higher concentration of hydrogen peroxide. The hydrogen peroxide and organic peroxyacid titration revealed that the proportion of hydrogen peroxide was higher in BioDestroy (17.3%) than organic peroxyacids (3.4 to 7.64%). Although hydrogen peroxide has been found to be ineffective against biofilms in other studies (42, 43), it seems to be able to penetrate *P. aeruginosa* biofilm and cause it to detach from its substratum (38). In the present study, hydrogen peroxide did affect the scanning electron microscopic appearance of *P. azotoformans* PFl1A biofilm formed on the peg (lack of the three-dimensional aspect of the biofilm), but the total detachment of the bacterial cells was not observed (Fig. 2). Furthermore, hydrogen peroxide treatment appeared to have a different effect from peracetic acid, perlactic acid, perpropionic acid, and BioDestroy (Fig. 2). In addition to the lack of the three-dimensional aspect of the biofilm, fewer cells remained attached to the pegs with the organic peroxyacids. However, biofilms were not totally removed from the pegs. Confocal microscopy confirmed that the cells attached to the pegs were dead, as evidenced by the mostly red-orange coloring of *P. azotoformans* within the biofilm treated with peracetic acid at 500 ppm (at the MBEC level) (Fig. 3). The fact that the biofilm did not completely detach from the surface could lead to further contamination by attracting live cells to its remaining portion during the following processing cycle and, thus, allowing the biofilm to persist.

Finally, the protective effect of a biofilm formed by *B. vesicularis* B40 against disinfectant and, thus, its ability to allow the persistence of pathogenic microorganisms such as *L. monocytogenes* were investigated. An antagonism assay was used to ensure that there was no interspecific inhibition between the isolates used in the mixed-species biofilms (data not shown). In the present study, *L. monocytogenes* 1046 was not significantly less sensitive to any disinfectant by virtue of being in a mixed-species biofilm with *B. vesicularis* B40 (Fig. 1). Moreover, the MBECs were similar for *L. monocytogenes* in a single-species biofilm as well as in a mixed-species biofilm with *B. vesicularis* B40 (Table 2). These results are of interest for the dairy industry, because *B. vesicularis* was the most frequently isolated microorganism from stainless steel equipment surfaces in Quebec dairy plants following detection of a probable biofilm issue. Several studies suggest that mixed-species biofilms are less sensitive to biocides than single-species biofilms (14, 15). In the present study, being in a biofilm coproduced with *B. vesicularis* 40 did not make *L. monocytogenes* 1046 more resistant to disinfectants. This is consistent with other studies that show how growing in a mixed-species biofilm is not always an advantage for microorganisms (44, 45).
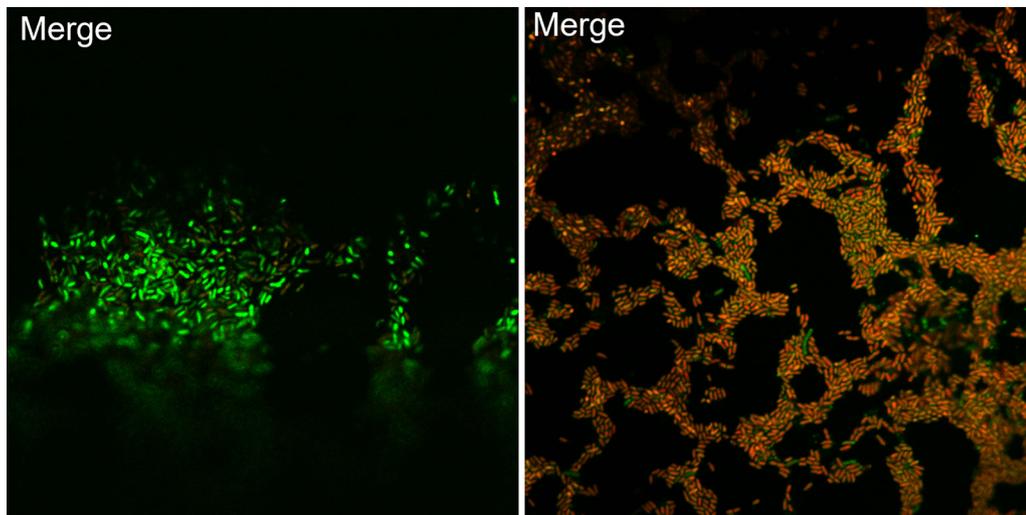
FIG 3 Viability of biofilm formed by *Pseudomonas azotoformans* PFl1A on MBEC assay peg before (left) and after (right) treatment with peracetic acid (500 ppm), visualized by confocal laser scanning microscopy (63×/1.40 oil differential interference contrast) with LIVE/DEAD staining (Invitrogen). Viable cells are green.

However, further research should be conducted on more isolates of *L. monocytogenes* to confirm this observation.

In conclusion, some of the isolates collected in Quebec dairy industries were moderate or strong biofilm producers using microtiter plates. Although these biofilms (single- and mixed-species biofilms) are sensitive to organic peroxyacids, dead bacterial cells remained attached to the surfaces, promoting the adhesion of other microorganisms. Microtiter plates are frequently used to rapidly screen the ability of microbes to form biofilms. Unfortunately, they are closed systems with no shear forces that are produced during milk processing. Therefore, to confirm our results, further studies should be carried out using a CDC bioreactor, a dynamic biofilm formation method. This method would also enable the use of stainless steel surfaces, which constitute most of the equipment in food and dairy industries. Furthermore, the combined use of organic peroxyacids, especially BioDestroy, with mechanical (vibrational, ultrasonic) or enzymatic treatments should also be evaluated to develop a more effective biofilm suppression strategy for the dairy industry.

MATERIALS AND METHODS

Microbial isolates and growth conditions. The microbial isolates used in this study are listed in Table 1. *Listeria monocytogenes* isolates (1045 and 1046) were obtained from Steve Labrie (Université Laval, Canada). The other isolates were obtained by swabbing stainless steel equipment surfaces suspected of containing biofilms in Quebec dairy plants. Biofilm formation on equipment surfaces is suspected when the contamination of processed dairy products persists even after additional cleaning cycles. Isolates were presumptively identified using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonik GmbH) (46). All isolates were cultured on tryptic-soy broth (TSB; BD Bacto number DF0370-17-3) and incubated with agitation (160 rpm) for 16 h or 48 h at 30°C.

Single-species biofilm assay. Bacterial biofilms were cultured in 96-well microtiter plates (Corning Costar number 3595) as described previously, but with modifications (44). Briefly, suspension culture (16 to 24 h) was diluted 1/100 or 1/1,000 in TSB to a concentration of approximatively 2×10^7 CFU/ml. Three wells were loaded with 200 μl of diluted culture each, and the plate was incubated at 37°C for 24 h without agitation. Planktonic cells were then eliminated by removing the liquid and washing the wells three times with phosphate-buffered saline (PBS). The biofilm was air dried, stained with 0.1% (wt/vol) crystal violet (Fisher Chemical number C581-25) for 10 min, decolorized with 200 μl of 50% (vol/vol) ethanol plus 50% (vol/vol) glacial acetic acid, and quantified by measuring absorbance at 570 nm. The ability of isolates to form a biofilm was classified as strong ($A_{570} \geq 1.5$), moderate ($0.5 \geq A_{570} < 1.5$), or weak/negative ($A_{570} \leq 0.5$). An arbitrary scale was used in this study to classify the various species. This scale was based on the one used for coagulase-negative staphylococci (41). Each experiment was repeated on three independent days.

Preparation of disinfectants. Peracetic, perpropionic, and perlactic acids were prepared as described previously (18). Briefly, acetic (Sigma-Aldrich number 27225, Food Chemicals Codex [FCC]), propionic (Laboratoire MAT number PF-0221, FCC), or lactic (Laboratoire MAT number LU-0200, FCC) acid solution was mixed with 30% hydrogen peroxide (Sigma number 216763) at an acid/peroxide volume ratio of 1.5, and a solution of 1% sulfuric acid was then added. The solutions were incubated at 30°C for 48 h and stored at −20°C until use. BioDestroy (Sani Marc Inc.), a commercialized antibiofilm product containing peracetic acid and hydrogen peroxide, was also tested. Hydrogen peroxide and organic peroxyacids were titrated in triplicate using a colorimetric reaction described previously by Greenspan and MacKellar (47). All disinfectant dilutions were made aseptically using sterile distilled water. The disinfectants were tested at concentrations of 0, 25, 50, 500, 1,000, 5,000, 10,000, and 25,000 ppm of active substance, except for hydrogen peroxide (0, 1,000, 10,000, 25,000, 50,000, and 100,000 ppm).

MBEC assay. The efficacy of disinfectants against dairy biofilms was tested using an MBEC assay biofilm inoculator with a 96-well base (Innovotech number 19111) as described by the ASTM, with modifications (48). Briefly, 150 μl of bacterial suspension, diluted as described above, was loaded into three wells and then incubated. For mixed biofilm assays, 75 μl of each suspension was added for a total volume of 150 μl. After the incubation, planktonic cells were removed by soaking the MBEC lid in PBS for 10 s. The MBEC lid was then transferred to a microtiter plate containing the disinfectant and incubated at room temperature for 5 min. A 5-min contact time is commonly used in the Canadian food industry, including dairy industry, by following the safety and efficacy requirements for hard surface disinfectant drugs by Health Canada (49). After the incubation, the MBEC lid was transferred to a microtiter plate containing a neutralizer, and the plate was sonicated for 30 min to detach the biofilm from the pegs. Microorganisms recovered from the single-species biofilms were quantified by diluting serially and spot plating on tryptic-soy agar medium (TSA). For mixed biofilms, microorganisms recovered were plated in TSA and in *Listeria* selective oxford agar base (Thermo Scientific number OXCM0856B).

The $\log_{10}$ density for each peg and the $\log_{10}$ reduction at each disinfectant concentration were calculated as

$$\mathrm{Log}_{10}\left(\mathrm{CFU}/\mathrm{mm}^2\right) = \mathrm{Log}_{10}\left[(X/B)\,(V/A)\,(D)\right]$$

where X is number of CFU counted in the spot, B is volume plated (0.01 ml), V is well volume (0.20 ml), A is peg surface area (46.63 mm²), and D is dilution.

$$\mathrm{Log}_{10}\ \mathrm{reduction} = \left(\mathrm{mean}\ \log_{10}\ \mathrm{untreated\ control\ pegs}\right) - \left(\mathrm{mean}\ \log_{10}\ \mathrm{treated\ pegs}\right)$$

Furthermore, the lowest disinfectant concentration that showed no bacterial growth was identified as the MBEC. Each experiment was repeated on three independent days.

Scanning electron microscopy. After being treated with the disinfectants, *P. azotoformans* biofilms formed on the MBEC pegs were fixed with 5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.5), covered with epoxy resin, and then visualized using a scanning electron microscope (JSM-6360LV model; JEOL) according to the MBEC assay procedural manual, version 2.1 (Innovotech). Images were acquired using the software provided by the manufacturer.

Confocal laser scanning microscopy. *P. azotoformans* biofilms formed on the MBEC pegs were treated with the disinfectants, stained using the LIVE/DEAD biofilm viability kit (Invitrogen) according to the MBEC assay procedural manual, version 2.1 (Innovotech), and then visualized by confocal laser scanning microscopy (LSM 700 confocal; Zeiss). Images were acquired using Zen software (Zeiss).

Statistical analysis. A two-way analysis of variance (ANOVA) followed by a Tukey multiple-comparison test (GraphPad Prism version 9.2.0 software) was used to compare the efficacy of the five disinfectants at different concentrations against the same isolate as well as the efficacy of the same disinfectant at different concentrations against the different bacterial isolates. Differences were considered statistically significant at a P value of <0.05.

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