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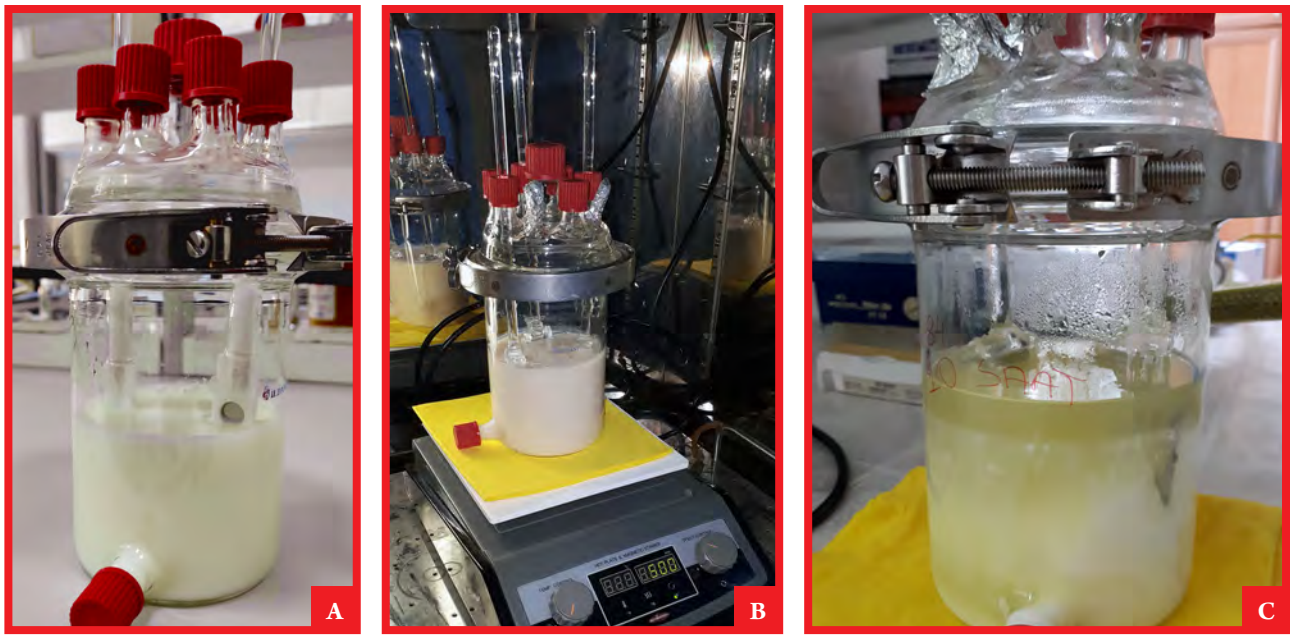

Before (a), during (b), and after (c) cultivition of thermophilic bacilli in a Skim Milk containing CDC biorector for biofilm formation process on coupons at 60°C up to 18h . At the end of the cultivation, biofilm containing abiotic surfaces were treated with Clean-in-Place Agents for biofilm removal.

Evaluation of Clean-in-Place Agents on the Removal of Thermophilic Biofilms Formed under Partially Simulated Conditions Associated with the Dairy Industry

ABSTRACT

In this study, various clean-in-place agents against thermophilic biofilms were evaluated. Dairy contaminants such as *Geobacillus thermodenitrificans, Geobacillus thermoglucosidans, Anoxybacillus flavithermus,* and *Anoxybacillus kamchatkensis* subsp. *asaccharedens* were sampled under simulated conditions (whole milk, static and dynamic conditions). Biofilm removal strategies were conducted, and agents acting on proteins such as trichloroacetic acid (TCA) and sodium dodecyl sulfate (SDS) were found to be most effective on thermophilic biofilms. It was found that thermophilic strains, especially under dynamic conditions, can form intense biofilms at an early stage (<5 h) and that these biofilms cannot be removed by the previously recommended routine sanitation procedures. In the current study, tandem treatment (SDS after TCA) with chemicals such as TCA and SDS for 30 min gave clear results in removing thermophilic biofilms. Moreover, enzymes such as trypsin and protease were highly effective in removing thermophilic biofilms. It was also found that surface materials used in the dairy industry, such as stainless steel, polypropylene, polyvinyl chloride, and polycarbonate, are not critical for the removal of thermophilic biofilms. Thermophilic biofilms sampled on surfaces under simulated conditions (whole milk, temperature, steady-state, and dynamic conditions) were also evaluated using confocal laser microscopy analysis after sanitary treatment.

INTRODUCTION

A microbial biofilm is a formation consisting of one or more species of microorganisms attached to an abiotic or biotic surface and surrounded by a self-producing matrix. The biofilm matrix is a network of polysaccharides, nucleic acids, and proteins synthesized by microorganisms that protect the microorganisms from harsh environmental conditions *(20)*.

Microorganisms are the main factors determining the reliability and quality of dairy products. The main source of continuous contamination of dairy products is the industrial facilities where the products are processed *(1, 16, 32)*. Many studies have shown that microorganisms can survive routine sanitation procedures because of their ability to form

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biofilms on product processing equipment *(18, 28)*. Even when equipment in the dairy industry is subjected to routine sanitation, microorganisms can remain on surfaces. As a result, bacteria can survive because they can form biofilms on the surface and cause continuous contamination of dairy products. Therefore, to identify effective sanitation strategies, it is important to study the microflora of dairy products and the ability of members of the microflora to form biofilms depending on the type of processing environment and various environmental factors *(27, 28)*. Once formed, biofilms significantly reduce the life and efficiency of processing systems, and biofilm bacteria can contaminate food products, leading to product spoilage and the spread of foodborne illness *(26)*.

In the evaporators of milk powder manufacturing plants, thermophilic bacilli such as *Geobacillus stearothermophilus* can form biofilms on surfaces where the bacteria can attach and where temperatures are approximately 55°C. Microorganisms can colonize surfaces of milk processing equipment even when previously recommended routine sanitation and disinfection procedures have been implemented *(8, 19)*. Thermophilic spores and bacteria capable of forming biofilms can be encountered on all surfaces of milk processing equipment, e.g., stainless steel surfaces *(23)*. Thermophilic bacilli are approved as hygiene indicators for processed products in the dairy industry. This phenomenon is related to the ability of thermophilic bacteria to form endospores and biofilms. In addition, thermophilic bacteria are potential spoilage agents because they can produce enzymes and acids that can reduce food quality *(18)*.

Control of microbial contamination in dairy plants usually requires a process called clean in place (CIP), which can also target biofilms of thermophilic bacilli. CIP processes differ in the removal of surface-adhering bacteria. The application time, temperature, concentration, and chemistry of the treatment as well as the characteristics of the surface to be cleaned are the main factors that determine the effectiveness of the CIP process *(3)*.

The main purpose of the current study was to investigate the impact of routine sanitary treatments on thermophilic biofilms sampled on different abiotic surfaces under both stationary and dynamic conditions. In addition, new strategies for the removal of thermophilic biofilms have been proposed in cases for which routine sanitation procedures are inadequate. The thermophilic bacilli included in the study are potent biofilm producers, and biofilm samples were sampled on various surface materials used in the dairy industry. Thermophilic biofilms developed on different types of surfaces were analyzed using confocal laser microscopy (CLSM) before and after sanitation. Various microscopic and biochemical methods have been developed and successfully used to describe biofilm formation. CLSM is an effective instrument for studying biofilms; it allows nondestructive and tophographic imaging of biofilms. In this study, the structures of thermophilic biofilms were investigated in microscopic detail, taking into account the partial simulation of conditions in their natural environment for the first time.

MATERIALS AND METHODS

Microbial strains

In this study, four endospore-forming thermophilic bacilli were preferred: *Geobacillus thermodenitrificans* DSM 465^T, *Geobacillus thermoglucosidans* B84a, *Anoxybacillus flavithermus* DSM 2641^T, and *Anoxybacillus kamchatkensis* subsp. *asaccharedens* F81 (Microbiology Research Laboratory, Ankara University, Turkey).

Culture preparation

Strains in glycerol stocks at -86°C were first inoculated into MI agar (medium 1; starch 10 g/liter, caso-peptone 5 g/liter, yeast extract 3 g/liter, meat extract 3 g/liter, K_2HPO_4 3 g/liter, KH_2PO_4 1 g/liter, agar 30 g/liter, pH 7.0 ± 0.2) and were incubated for 24 h at 55°C. After this step, the preactivation steps mentioned below were performed before all biofilm assays. Colonies grown on MI agar plates were harvested and transferred to 3 ml of tryptic soy broth (TSB; Merck, Darmstadt, Germany). The TSB cultures were incubated for 18 h at 55°C (170 rpm). At the end of the incubation, the active cultures were again inoculated into 5 ml of TSB without NaCl and incubated for another 6 h at 55°C (170 rpm). The appropriate inoculation procedure ensured that the thermophilic bacilli to be inoculated remained in vegetative cell form by preventing the transition to the sporulation phase prior to biofilm assays *(14)*.

Biofilm sampling under static conditions and effects of CIP agents

Ideal abiotic surfaces were preferred based on a previous study for each thermophilic strain *(Table 1)* *(12)*. The preferred surfaces are the best for intensive biofilm formation of the thermophilic bacilli tested.

Biofilm sampling on selected surfaces (radius, 7 mm; total surface area, 3.08 cm^2) was performed in six-well polystyrene tissue culture plates. Two of the surfaces were transferred to wells for each strain. Sterile reconstituted skim milk (5 ml; 10% powdered milk, Sigma-Aldrich, St. Louis, MO) inoculated with 4% inoculum (inocula were prepared as described above) was transferred to each well. The plates were incubated for 48 h at 60°C. At the end of the first 24 h, the contents in the wells were renewed and the plates were incubated for another 24 h at 60°C. The 48-h incubation time was the best to obtain a sufficient amount of biofilm for all tested thermophilic bacteria under static conditions (data not shown).

After incubation, biofilm samples were rinsed twice with physiological saline (0.85% NaCl) to remove planktonic counterparts. The rinsed surfaces were then transferred to plates. The surfaces were treated with various sanitizers as indicated in *Table 2*.

After appropriate treatment with each agent, surfaces were rinsed twice with physiological serum to remove chemical and enzyme residues. The treated surfaces were then transferred to glass tubes containing 5 ml of physiological serum and 10 g

TABLE 1. Selected surfaces for biofilm sampling *(12)*

G. thermodenitrificans DSM 465[T]		*G. thermoglucosidans* B84a	
316 L Stainless steel	Polycarbonate	316 L Stainless steel	Polycarbonate
***A. kamchatkensis* subsp. *asaccharedens* F81**		***A. flavithermus* DSM 2641[T]**	
316 L Stainless steel	Polypropylene	PVC	Polycarbonate

TABLE 2. CIP agents used in this study

Sanitation agents	Brands and CAT no.	From literature	From study	Buffer	Treatment temp	Contact time	Reference
Agents that affect carbohydrates							
Sodium metaperiodate	Sigma 71859	50 mM	100 mM	dH_2O	22°C	60 min	*(23)*
Agents that cause precipitation of macromolecules							
Trichloroacetic acid	Sigma 27242	10%	10%	dH_2O	100°C	15 min	*(23)*
Agents that cause membrane lysis							
Lysozyme	Sigma L-7651	2%	2%	10 mM Tris-HCl	37°C	60 min	*(23)*
Agents that cause protein denaturation							
Sodium dodecyl sulfate (SDS)	Sigma L-5750	2%	3%	dH_2O	100°C	10 min	*(23)*
Trypsin	Sigma T2600000	2%	3%	50 mM Tris-HCl	37°C	3 h	*(23)*
Pro-oxidants							
Potassium monopersulfate (PMS) Sodium thiosulfate	Sigma 228036 Sigma 72049	1 mg/ml 9 mg/ml	2 mg/ml 10 mg/ml	SF (serum physiologic 0.85% NaCl in dH_2O)	22°C 22°C	30 min 5 min	*(23)*
Antibacterial and bacteriocidal agent							
Nisin	Sigma N5764	1 mg/ml	2 mg/ml	0.02 N HCl	37°C	24 h	*(2)*

Continued on next page

TABLE 2. CIP agents used in this study (cont.)

Sanitation agents	Brands and CAT no.	From literature	From study	Buffer	Treatment temp	Contact time	Reference
Enzyme-based cleaning agents							
a. Protease agents							
Alkaline protease	Sigma P4860	0.08%	0.16 U/g	50 mM glycine-NaOH	60°C	60 min	*(24)*
Subtilisin	Sigma P5380	0.5%	1%	0.1 M borate, 0.1 M $CaCl_2$	37°C	30 min	*(24)*
b. Polysaccharide-degrading agents							
α-Amylase	Sigma A4551	0.5%	1%	20 mM sodium phosphate, 6.7 mM NaCl	37°C	30 min	*(24)*
Cellulase	Sigma C1184	0.3%	1.66%	50 mM sodium acetate	37°C	30 min	*(24)*
Agents that cause inhibition of quorum sensing							
a. Inhibition of signal receptors							
Furanon	Sigma 283754	1 mg/ml	1 mg/ml	dH_2O	22°C	60 min	*(25)*
b. Inhibition of signal molecule production							
Triclosan*	Sigma PHR1338	1 mg/ml	2 mg/ml	50% ethanol	22°C	60 min	*(30)*
0.5 M NaCl	Merck 1.06404	0.5 M	0.5 M	dH_2O	22°C	30 min	*(24)*
1.0 M NaCl	Merck 1.06404	1.0 M	1.0 M	dH_2O	22°C	30 min	*(24)*

of glass beads (radius, 1.5 mm). The remaining biofilms were harvested by vortexing the surfaces at maximum intensity for 2 min (IKA vortex, IKA, Staufen, Germany). The biofilm suspensions were diluted 10-fold, and serial dilutions were dropped onto tryptic soy agar (TSA) plates (Merck) to calculate total colony counts (both vegetative cells and spores). TSA plates were incubated for 24 h at 60°C. Untreated surfaces were used as positive control groups. The total number of CFU was divided by the total surface area to calculate the units per cm^2 of surface area. Subsequently, the divided values were converted to a logarithmic scale. Log reduction was calculated by subtracting test group values from positive control values. The percentage of log reduction was also calculated using the formula given: $(1 - 10^{-LR}) \times 100$; where LR is the log reduction. The biofilm suspensions remaining after the total colony count were also used for the spore count. The suspensions were boiled for 15 min to eliminate vegetative cell forms. The boiled suspensions were counted, and the log reduction values were calculated as described previously *(12)*.

Biofilm sampling under dynamic conditions and effects of ideal CIP agents

A modified Centers for Disease Control and Prevention (CDC) reactor, a frequently preferred system for sampling biofilms under dynamic conditions, was used in the study *(Fig. 1)*.

Figure 1. (a) The modified CDC reactor. (b) The holders placed with ideal abiotic surfaces.

The assembled reactor was treated with 1.2% sodium hypochlorite and autoclaved after it was rinsed with distilled water *(22)*. The reactor was filled with 576 ml of sterile 10% reconstituted skim milk. The sterilized abiotic surfaces were placed in reactor holders, and the holders were then transferred to the reactor. Finally, the entire volume of reconstituted skim milk was inoculated with the active culture (4% inoculum ratio). The reactor was then transferred to an incubator with a magnetic stirrer. Incubation was performed at 60°C for 2 and 18 h (*Geobacillus* strains) and 5 and 18 h (*Anoxybacillus* strains). Biofilm formation was accelerated at 2 h for *Geobacillus* strains and at 5 h for *Anoxybacillus* strains *(13)*. For a sufficient amount of biofilm samples, 18 h was ideal. Longer incubation times were unsuitable for CLSM analysis due to the accumulation of thick biofilms on surfaces (unpublished data). Biofilm collection, sanitizer treatment, and counting procedures were performed as previously described.

CLSM imaging

The treated and untreated surfaces were stained with 5 µM SYTO 9 (S34854, Molecular Probes, Eugene, OR) and 15 µM propidium iodide (PI) solutions (P1304MP, Molecular Probes) after rinsing. The surfaces were incubated at 30°C in a dark environment for 20 min and were washed twice with sterile distilled water *(5, 9)*. SYTO 9 fluorescent stain (excitation, 488 nm; emission, 498 nm) interacts directly with living cells and emits green fluorescent light, whereas PI (excitation, 543 nm; emission, 617 nm) is absorbed by the dead cells and gives them fluorescence properties in the red spectrum *(31)*. PI and SYTO 9 also allow extracellular DNA in the biofilm matrix to appear with a yellowish color *(21)*. Stained samples were stored at 4°C in a dark environment until they were observed using CLSM *(15)*. In the CLSM study, clean surfaces that served as negative controls were also stained with SYTO 9 and PI.

The treated and untreated biofilm samples were examined using CLSM (Zeiss LSM 510) and Plan-Neofluar 40X/1.3 differential interference contrast objectives (both from Carl Zeiss, Jena, Germany). Because milk residues also react with dyes, the green fluorescence of SYTO 9 was excited with an argon laser source at 540 nm and the red fluorescence of PI was excited with a helium or neon laser source at 660 nm. Fluorescence was recorded in bandpass 505 to 530 nm and longpass 650 nm with 30% laser power in two diachronic mirrors to separate the two laser sources. Microscopic fields were determined in each specimen with 2D (*x*-axis or *y*-axis only, 230.34 × 230.34 µm) or 3D (*x*, 230.34 µm; *y*, 230.34 µm; *z*-axis, 4 µm) horizontal plane images. Live and dead cell densities were determined, and 3D biofilm thicknesses (µm) were also measured.

RESULTS AND DISCUSSION

Eradication of thermophilic biofilms sampled under static and dynamic conditions

The eradication results for all thermophilic biofilms are presented in *Supplementary Tables S1* to *S3*; the results are also summarized in *Table 3*.

In particular, the biofilms of *A. kamchatkensis* subsp. *asaccharedens* F81 sampled on stainless steel and polypropylene surfaces could not be eliminated by any of the tested chemical and enzymatic sanitizers *(Table 3)*. The biofilm cells and spores of *A. flavithermus* DSM 2641T, which causes problems with its biofilms in the dairy industry, were 100% removed by treatment with alkaline protease and SDS. In addition, biofilm spores were removed from polyvinyl chloride and polycarbonate surfaces with TCA and triclosan. Both biofilm cells and spores on polyvinyl chloride surfaces were removed with sodium metaperiodate alone, but removal rates on polycarbonate surfaces remained lower for this strain *(Table 3)*.

The biofilm cells of *G. thermodenitrificans* DSM 465^T on stainless steel and polycarbonate surfaces could not be 100%

TABLE 3. Log reduction values obtained from sanitary treatment (The most effective results were highlighted with different colors, >98.0%)

	G. thermodenitrificans DSM 465T				*G. thermoglucosidans* B84a				*A. kamchatkensis* subsp. *asaccharedens* F81				*A. flavithermus* DSM 2641T			
	Stainless steel		Polycarbonate		Stainless steel		Polycarbonate		Stainless steel		Polypropylene		PVC		Polycarbonate	
	Biofilm	Spore	Biofilm	Spore	Biofilm	Spore	Biofilm	Spore	Biofilm	Spore	Biofilm	Spore	Biofilm	Spore	Biofilm	Spore
Subtilisin	99.92	99.75	99.11	13.04	99.85	99.29	99.51	86.20	84.79	83.44	61.90	72.50	21.67	92.25	65.00	26.25
Alkaline protease	99.92	99.75	99.89	99.83	99.83	96.43	99.76	94.43	77.08	81.25	48.57	74.50	100	100	100	99.92
Cellulase	23.12	65.83	78.22	1.30	98.54	96.43	99.50	95.82	62.50	76.88	87.62	89.00	73.33	75.00	35.00	75.00
α-Amylase	99.92	99.13	99.76	98.13	84.62	97.50	98.31	99.51	80.21	75.00	68.57	60.00	33.33	20.00	98.00	92.50
Trypsin	99.99	100	99.99	100	99.98	100	100	99.95	99.71	99.75	97.14	97.35	98.33	98.75	95.00	95.00
SDS	99.19	91.38	87.67	96.96	99.85	100	99.74	92.78	98.69	99.41	98.48	98.80	100	100	100	100
TCA	99.23	96.67	97.94	93.91	100	100	99.70	100	92.92	99.99	99.93	99.95	99.50	100	99.97	100
Furanon	87.69	99.92	98.00	88.26	99.56	99.64	99.31	92.41	54.17	37.50	60.00	66.00	76.67	92.50	60.00	97.50
Triclosan	99.99	99.92	99.84	99.83	99.91	100	93.13	65.82	69.17	80.00	47.14	80.00	99.83	100	99.00	100
Lysozyme	99.23	98.25	99.99	99.91	98.46	94.11	99.99	99.97	99.98	99.99	80.95	88.00	98.33	99.75	50.00	0.00
0.5 M NaCl	23.08	98.33	0.56	96.52	97.85	99.64	98.66	87.59	86.46	47.50	4.76	55.00	50.00	50.00	15.00	25.00
1.0 M NaCl	95.38	99.92	44.44	97.70	99.95	99.29	99.76	90.51	85.83	69.69	63.81	76.00	66.67	75.00	50.00	75.00
PMS-Sodium thiosulfate	99.92	99.92	97.39	100	99.97	99.64	98.09	85.95	66.67	68.75	64.76	91.80	63.33	97.00	45.00	97.50
Sodium metaperiodate	99.95	100	99.98	99.91	99.72	99.29	99.77	98.11	89.79	91.88	58.10	71.00	100	100	98.50	95.00
Nisin	99.98	99.99	99.97	99.98	99.23	89.29	99.38	99.49	99.98	99.99	99.92	99.95	50.00	45.00	35.00	75.00

removed by any agent, but the biofilm spores on these two surfaces could be 100% eliminated after trypsin treatment. Although the biofilm spores of *G. thermoglucosidans* B84a on both stainless steel and polycarbonate surfaces could be completely removed by trypsin treatment, the biofilm spores on stainless steel were eradicated by SDS and triclosan treatments. In addition, the biofilms of this strain on stainless steel were removed with TCA *(Table 3)*.

The success of chemical and enzymatic agents for biofilm eradication varied depending on the incubation period, surface characteristics, bacterial strains, and components of the biofilm matrix. The success of biofilm eradication described above was possible with thermophilic biofilms sampled under static conditions. However, dairy products are processed in dynamic environments, and eradication of biofilms that develop in these environments is more critical. For this reason, the next study was conducted using agents that provide at least >98.0% eradication. Preferred agents were lysozyme (98.25 to 99.99%) for *G. thermodenitrificans* DSM 465^T; sodium metaperiodate (99.91 to 100%), trypsin (99.95 to 100%), and TCA (99.7 to 100%) for *G. thermoglucasidans* B84a; SDS (100%) and TCA (99.5 to 100%) for *A. flavithermus* DSM 2641^T; and SDS (98.48 to 99.41%) and TCA (92.92 to 99.99%) for *A. kamchatkensis* subsp. *asaccharedens* F81 (*Table 4* and *Tables S1* to *S3*).

The tested sanitation agents successfully removed biofilms sampled under static conditions at high rates; this result was not achieved for the removal of biofilms sampled under dynamic conditions (*Fig. S1* to *S4*).

Considering all these findings, a final eradication study was designed, and the simultaneous application of sanitizing agents such as SDS and TCA (treatment at 100°C for 10 and 15 min, respectively) was tested to remove all thermophilic biofilms *(Fig. 2)*.

The 18-h-old biofilms of DSM 465^T, B84a, and DSM 2641^T strains were removed by application of two selected sanitizing agents. Biofilm cells and spores were removed on the stainless steel surface, but removal rates on the polypropylene surface remained at 99.5 and 96.7% for strain F81, respectively *(Fig. 2)*.

CLSM analysis

Prior to CLSM analysis, incubation times for biofilm sampling of strains were shorter (5 and 18 h at 60°C for *Anoxybacillus*, 2 and 18 h at 60°C for *Geobacillus*) due to intensive biofilm production under dynamic conditions. Older biofilm samples (>18 h) could not be properly examined in CLSM imaging due to increased biofilm thickness (data not shown). Biofilms sampled under dynamic conditions were much thicker and accumulated intensively on the surfaces despite a short incubation period *(Fig. 3)*.

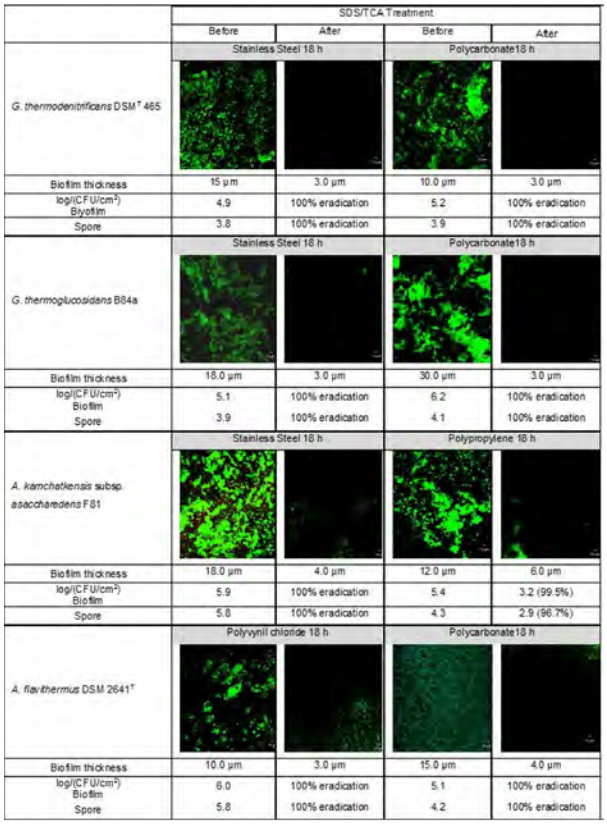

Figure 2. Eradication with combined treatment of SDS and TCA of 18-h biofilms of DSM 465^T, B84a, F81, and DSM 2641^T (biofilms sampled under dynamic conditions).

CLSM images of thermophilic biofilms sampled in milk are illustrated in *Figure 3*. They are 3D projections of 18-h-old biofilms formed under dynamic conditions for strains *G. thermodenitrificans* DSM 465^T, *A. flavithermus* DSM 2641^T, and *A. kamchatkensis* subsp. *asaccharedens* F81.

CLSM image sections showed cells in vegetative form, endospores, microcolonies, and extracellular DNA (eDNA, yellow fluorescence) stained with SYTO 9. Dead biofilm cells were also observed after lysozyme treatment.

From the CLSM analysis, it was clear that the 2-h-old biofilms of *Anoxybacillus* and the 5-h-old biofilms of *Geobacillus* were formed in a short time on ideal abiotic surfaces. A significant increase in the thickness of the biofilms was observed at the end of the 18-h incubation period. Moreover, there were changes in biofilm thickness and matrix composition depending on the bacterial strain and abiotic surface. In particular, the results of the 3D horizontal projection on the z-axis showed that 2-h-old biofilms consisted of living cells with low matrix content, whereas 18-h-old biofilms contained eDNA that emitted intense fluorescence. Increased red fluorescence was observed when biofilm layers were examined near the substrate. Upper layers of biofilms contained a large number of living cells (green fluorescence) (*Fig. S1* to *S4*). This microscopy technique provided extremely useful data for before and after the sanitation process because it allowed the biofilm samples to be examined without destruction. Moreover, it was observed for the first time that biofilm samples from a dense organic and inorganic medium such as milk could be analyzed by fluorescent staining and CLSM.

Hinton et al. *(10)* demonstrated that caustic solutions used in cleaning equipment cannot eliminate most bacteria in product residues. CIP procedures in the food industry often eliminate problems that may be caused by food pathogens, but they may not be effective for thermophilic bacilli that negatively affect food quality *(23)*. Polymeric components (proteins, carbohydrates, fatty acids, nucleic acids, cations, anions, etc.) that are released by microbial consortia into the extracellular environment and that establish the structural or functional integrity of the biofilm make biofilm cells more resistant to sanitary agents than their planktonic counterparts *(11)*. The protein, carbohydrate, and nucleic acid content of thermophilic biofilms was determined prior to the corresponding study (data not shown). In this context, the study also focused on the evaluation of sanitation processes based on extracellular polymeric components.

The efficacy of polysaccharidases and proteolytic enzymes against biofilms formed in product processing lines was tested by Lequette et al. *(17)*. Serine protease and α-amylase were found to be the most effective enzymatic agents against biofilms. Proteolytic enzymes have a broader spectrum compared to polysaccharidases and are more effective in removing biofilms; in particular, serine proteases are extremely effective on *Bacillus* biofilms. Polysaccharidases are more effective in removing *Pseudomonas* biofilms. Enzyme solutions containing surfactants and chelating agents have also been reported to give better results in removing *Bacillus* and *Pseudomonas* biofilms *(6)*.

Parkar et al. *(24)* studied the removal of *A. flavithermus* biofilms with cleaning agents in place. They found that it is important to apply sanitizing agents at ideal concentrations and temperatures and also that the tested chemical agents were not sufficient to remove thermophilic biofilms on surfaces. Parkar et al. *(24)* also showed that polysaccharides in biofilm structures can only be degraded by caustic treatment at temperatures of 60°C and above. It was observed that the biofilm of *A. flavithermus* was eradicated only by acid and alkaline treatment applied at 75°C for 30 min. However, in our study, it was possible to remove 100% of the *A. flavithermus* biofilm in a shorter time using selected sanitation strategies *(Table 3)*. The sanitation agents tested in our study, mainly SDS and TCA, are the most successful agents for removing all thermophilic biofilms. The sanitation strategies evaluated in this study were selected based on the biochemical composition of the thermophilic biofilms studied. The effectiveness of sanitation strategies was evaluated considering both biomass (biofilm matrix) removal from the surface and biofilm cell killing. Although biofilm cells can be killed using sanitation strategies,

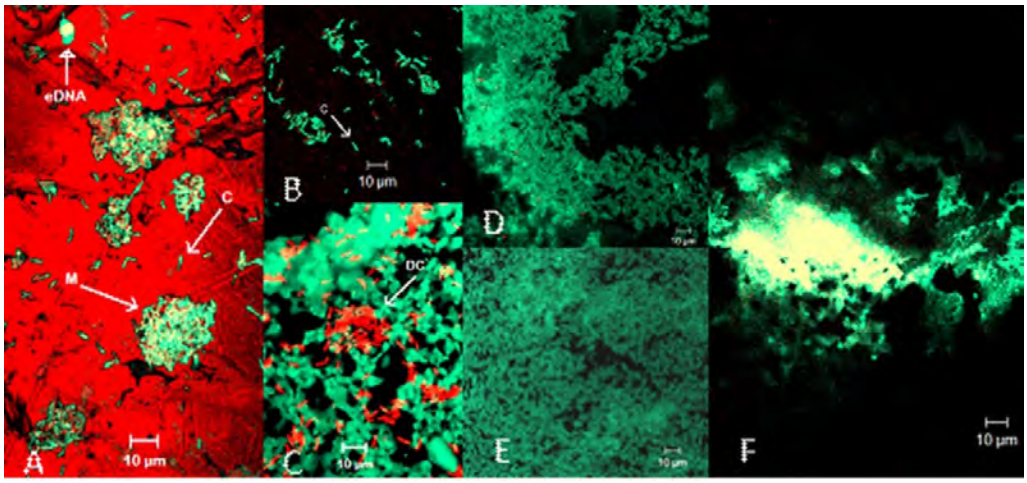

Figure 3. 3D CLSM sections of thermophilic biofilms developed for 18 h at 60°C under dynamic conditions (green fluorescence, live cells; red fluorescence, dead cells; yellow fluorescence, eDNA). (A) Biofilm formed by *G. thermodenitrificans* DSM 465^T on stainless steel (SS) surface. Live cells and eDNA content embedded in matrix (M, microcolonies; C, cell with endospores). (B) Vegetative cells in the biofilm matrix of DSM 465^T. (C) Dead cells (DC) in biofilm matrix (DSM 465^T) after lysozyme treatment. (D) 18-h-old biofilm of *A. flavithermus* DSM 2641^T on polyvinyl chloride (PVC) surface. (E) Live cells in the biofilm matrix of *A. flavithermus* DSM 2641^T on polycarbonate (PC) surface. (F) Biofilm of *A. kamchatkensis* subsp. *asaccharedens* F81 on polypropylene surface.

TABLE 4. The most effective chemical and enzymatic agents selected against biofilms sampled under dynamic conditions

Strain	Selected agents	Treatment conditions
G. thermodenitrificans DSM 465^T	2% lysozyme	37°C, 60 min
	100 mM sodium metaperiodate	22°C, 60 min
G. thermoglucosidans B84a	10% TCA	100°C, 15 min
	3% trypsin	37°C, 3 h
A. kamchatkensis subsp. *asaccharedens* F81	3% SDS	100°C, 10 min
	1 mg/ml nisin	37°C, 24 h
A. flavithermus DSM 2641^T	10% TCA	100°C, 15 min
	3% SDS	100°C, 10 min

the inability to remove organic and inorganic components in the matrix composition facilitates the attachment of new colonizers to surfaces. Protein components are more dominant in the biofilm composition of thermophilic bacilli used in our study (unpublished data), so more satisfactory biofilm eradication results were obtained with the use of agents such as TCA, SDS, trypsin, and alkaline protease (subtilisin) *(32)*. Caustic (alkaline) wash, the preferred sanitation method in the dairy industry, allows the removal of protein and carbohydrate residues *(29)*.

It is important to understand the nature of thermophilic biofilms and choose appropriate strategies to address the problems that thermophilic bacilli and biofilms can cause in the dairy industry. It appears that the biofilm removal capacities of protease and cellulase are similar to those of nonenzymatic agents such as SDS and TCA. Cellulase enzyme treatment showed the highest efficacy against the biofilm of *A. kamchatkensis*-subsp. *asaccharedens* F81 (89%) and *G. thermoglucosidans* B84a (99.5%), which had high carbohydrate content. Enzymes such as alkaline protease and amylase, which can successfully eradicate thermophilic biofilms, may be active under extreme conditions (high temperature, alkaline environment). Considering the high temperatures at which dairy products are processed, it is appropriate to prefer thermostable enzymes to remove thermophilic biofilms.

In cases for which routine hygiene practices may be inadequate, changes should be made that do not endanger human health or compromise food quality. To identify new hygiene strategies and address current problems, it is necessary to clarify the biofilm formation characteristics of thermophiles with laboratory-scale studies that mimic production environments in the dairy industry.

Under high shear stress, bacteria adhere more firmly to surfaces and form more durable biofilm structures than under low shear stress *(4, 7)*. Both laminar and turbulent shear forces allow bacteria to be closer to the surface and adhere more easily than under static conditions *(4)*. Biofilms sampled under dynamic conditions appear to form a much thicker and denser matrix compared to biofilm samples under static conditions. As a result, it was clarified that the preferred sanitation agents in the study are not adequate for biofilms developing under dynamic conditions. Although some sanitation strategies successfully removed cells and spores in biofilm structures that developed under static conditions, the same results could not be obtained for thermophilic biofilms sampled in the reactor.

The efficacy of some of the sanitation procedures applied at the point where biofilm formation is accelerated was demonstrated by data from microbiological counts and CLSM studies (5 h for *Anoaxbacillus*, 2 h for *Geobacillus*). In addition, it became clear that biofilms developing under dynamic conditions are difficult to eradicate and that sanitation procedures recommended for the dairy industry may be inadequate. Although the evaluated sanitation processes were completely effective in the early stages when biofilm production began, they were inadequate for mature biofilm samples. Another important finding of the study was that the physicochemical properties of the surfaces do not determine the success of sanitation.

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