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ORIGINAL PAPER

Biofilm-Forming Ability and Effect of Sanitation Agents on Biofilm-Control of Thermophile *Geobacillus* sp. D413 and *Geobacillus toebii* E134

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Abstract

Geobacillus sp. D413 and *Geobacillus toebii* E134 are aerobic, non-pathogenic, endospore-forming, obligately thermophilic bacilli. Gram-positive thermophilic bacilli can produce heat-resistant spores. The bacteria are indicator organisms for assessing the manufacturing process's hygiene and are capable of forming biofilms on surfaces used in industrial sectors. The present study aimed to determine the biofilm-forming properties of *Geobacillus* isolates and how to eliminate this formation with sanitation agents. According to the results, extracellular DNA (eDNA) was interestingly not affected by the DNase I, RNase A, and proteinase K. However, the genomic DNA (gDNA) was degraded by only DNase I. It seemed that the eDNA had resistance to DNase I when purified. It is considered that the enzymes could not reach the target eDNA. Moreover, the eDNA resistance may result from the conserved folded structure of eDNA after purification. Another assumption is that the eDNA might be protected by other extracellular polymeric substances (EPS) and/or extracellular membrane vesicles (EVs) structures. On the contrary, DNase I reduced unpurified eDNA (mature biofilms). Biofilm formation on surfaces used in industrial areas was investigated in this work: the D413 and E134 isolates adhered to all surfaces. Various sanitation agents could control biofilms of *Geobacillus* isolates. The best results were provided by nisin for D413 (80%) and α-amylase for E134 (98%). This paper suggests that sanitation agents could be a solution to control biofilm structures of thermophilic bacilli.

Key words: *Geobacillus* sp., abiotic surfaces, biofilm, sanitation agents

Introduction

Bacteria display two modes of growth: free-living planktonic or the sessile and surface-attached within biofilms (Rumbaugh and Sauer 2020). The bacteria colonize by adhering to surfaces, growing, and forming a self-produced polymeric matrix in which microbial species may grow together as a biofilm (González-Rivas et al. 2018). Biofilm growth is observed in many industrial and indigenous areas such as dairy, water systems, maritime, dentistry, food, paper, oil, optics, and healthcare fields (Garrett et al. 2008). One of the most prevalent biofilm-forming microorganisms in dairy manufacturing is the thermophilic bacilli (Burgess et al. 2013). The presence of the bacteria is an indicator of poor plant hygiene (Burgess et al. 2013). *Geobacillus* sp. is among the most widespread contaminants of milk powders. The bacteria survive during industrial pasteurization of milk, and spores adhere to surfaces and germinate to constitute biofilms, thus resulting in spoilage of milk products (Gopal et al. 2015). In addition, *Geobacillus stearothermophilus* strains were isolated from a milk powder manufacturing plant (Burgess et al. 2013). Simultaneously, members of the *Geobacillus* genus were isolated from hot springs, geothermal soil, composts, and water (Mandic-Mulec et al. 2015). *Geobacillus toebii* was first isolated by Sung et al. (2002) from hay compost.

Biofilms have become problematic in a wide range of food industries (González-Rivas et al. 2018). Examples of biofilms' harmful effects are product spoilage, reduced production capacity, corrosion, equipment failure, pipe blockages, and infection (Garrett et al. 2008). Spoilage bacteria are responsible for nearly one-third of losses in the food chain supply (González-Rivas et al. 2018). Biofilm formation of the bacteria in food manufacturing concerns the dairy industry (Lindsay and Flint

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2009). The reason behind is that thermophilic bacilli are hard to remove due to their broad temperature range of growth; they have a fast growth rate; their spores show high resistance to heat and chemicals; and they can form biofilms (Scott et al. 2007; Burgess et al. 2009; Eijlander et al. 2019). Eijlander et al. (2019) reported that *Geobacillus* spp. was identified after a heat treatment at 100°C for 30 min. Therefore, it is vital to remove the biofilms formed by the bacteria. The efficient cleaning of bacterial biofilms includes a combination of detergents, mechanical action, and sanitation agents. These regimes still appear to be the most effective way of combating *Bacillus* spp. biofilms. Biofilm control of thermophilic spore-forming bacilli can be achieved by temperature manipulation to limit growth, developing cleaning/sanitation, and treating surfaces to prevent attachment (Lindsay and Flint 2009). The cleaning processes involve the clean-in-place (CIP) system. The CIP regimes show variability in eliminating surface adherents. The cleaning chemicals play a significant role in the regime. They are based on firstly to decrease the surface population of bacteria thorough cleaning with detergent formulations, and then to kill the residual population through the application of disinfectants (Bremer et al. 2009). The primary strategy to eliminate biofilm formation is to clean and disinfect surfaces routinely before bacteria attach tightly (Shemesh and Ostrov 2020). Enzymes form an alternative for biofilm control, break up the biofilm matrix components, cause cell lysis, support biofilm degradation, and interrupt the cell-to-cell signaling (Meireles et al. 2016). Enzymatic solutions can be an eco-friendly, greener, and safe alternative for biofilm removal in the food industry (Mazaheri et al. 2020). Enzymes, including proteases, lipases, cellulases, and DNases, are frequently used (Ripolles-Avila et al. 2020). However, bacteria may be resistant to conventional treatments. Therefore, there is a need to enhance the methods and consider new strategies due to bacterial resistance's serious problem (Ripolles-Avila et al. 2019).

In our previous studies, we carried out the preliminary biofilm experiments, including pellicle formation, complex exopolysaccharide production, biofilm morphotypes, and viable biofilm cell counting on stainless steel of *Geobacillus* sp. D413 and *G. toebii* E134. We determined that the isolates were strong biofilm producers by the crystal violet binding assay (Cihan et al. 2017). The purpose of this study was to investigate the biofilm-forming abilities of *Geobacillus* strains on different abiotic surfaces and the effects of sanitation agents on the control of biofilm formation. In addition, it was aimed to screen the removal of the biofilms with DNase I, RNase A, and proteinase K enzymes and to determine the presence of eDNA in the biofilm matrix. There are very few research articles on this topic related to *Geobacillus* strains.

Experimental

Materials and Methods

Bacteria strain and culture conditions. *Geobacillus* sp. D413 was isolated from a soil sample from the hot spring (Dikili, Camur Hot Spring, Izmir, Turkey) and *G. toebii* E134 was isolated from a branch of a tree in the hot spring (Altinsu, Kozakli, Nevsehir, Turkey). The sequences of these isolates' 16S rRNA gene were registered with GenBank Accession Number FJ430040 and EU477771 for D413 and E134, respectively (Cihan et al. 2011). The isolates were primarily cultured in Tryptic Soy Agar (TSA, Merck, Germany) for 18 h at 55°C and then incubated in Tryptic Soy Broth (TSB, Merck, Germany) in a shaking incubator (170 rpm) at 55°C for 18 h and 6 h, respectively. All biofilm assays were carried out with the culture that was 6 h old in the mid-exponential growth phase, and the presence of non-sporulating vegetative cells was confirmed with phase-contrast microscopy. The inoculation process was essential to accelerate thermophilic bacilli's biofilm production capabilities by delaying their transition to the sporulation phase. The process was substantial to biofilm formation by endospore-forming thermophilic bacilli.

Effect of environmental conditions for bacterial growth. This assay was to study the influence of pH, salinity (sodium chloride, Merck, Germany), and temperature on the planktonic growth and biofilm formation. The optimal planktonic and biofilm growth of these isolates was determined at various ranges of pH (4.0 to 11.0), salinity (0 to 5%), and temperature (50 to 65°C) in 96-well polystyrene microtiter plates (LP, Italy). The bacterial growth was monitored spectrophotometrically (OD_{595} nm) at 0, 6, 18, 24, and 48 h in TSB. Furthermore, the biofilm growth was assayed with the crystal violet staining method at the end of the 48-hour incubation period. The optimal biofilm growth values were used in all other experiments. The negative controls contained only TSB.

Investigation of biofilm formation with crystal violet. The biofilm-forming ability of D413 and E134 on 96-well polystyrene microtiter plates was determined using the method of Woodward et al. (2000) and Stepanović et al. (2000) with some modifications. The wells were filled with 10 µl of culture and 90 µl of TSB without salt. The plate wells were cleaned two times with physiological saline at the end of 48 h of incubation to eliminate planktonic cells. The remaining adherent bacteria were fixed with 95% methanol (Merck, Germany) (200 µl) and incubated at room temperature. After that, the plates were emptied and air-dried. The wells were stained using 1% crystal violet (CV, Merck, Germany) for 30 min at 22°C. The plates were rinsed off with running tap water to remove the stain's surplus

and were air-dried. The dye bound to the biofilm cells was dissolved with ethanol: acetone (Merck, Germany). Finally, CV was quantified at an optical density (OD) of 595 nm using a microplate reader (BioTek Elisa reader, μQuant, Biotek Inc., USA). The negative controls contained only TSB.

Determination of the molecular weight of gDNA and eDNA. The bacterial biomass was collected after 18 h of incubation from the TSA. The biomass was dissolved with physiological saline and centrifuged. The pellet was used for gDNA isolation. The supernatant was filtered (0.22 μm membrane filter, Sartorius, France) and used for eDNA isolation. Extracellular DNA isolation was conducted partially by the method described by Wilson (2001), while gDNA isolation was done with a gDNA purification kit (Fermentas K0512, Thermo Fisher Scientific Inc., USA). Thus, gDNA and eDNA were partially purified. Ultimately, gDNA and eDNA values were measured at the absorbance values of 260 nm/280 nm with a Nanodrop spectrophotometer (Thermo Scientific NanoDrop Lite, USA). The samples were subjected to 1.5% agarose gel electrophoresis at 120 V for 45 min. After agarose gel electrophoresis, DNA products were visualized via a Quantum ST4 Gel Documentation System (Vilber Lourmat, France). The molecular weights of DNA samples were determined with the help of the Quantum-Capp software system (Vilber Lourmat, France).

Treatment of partially purified gDNA and eDNA with enzymes. In this assay, 10 μl of partially purified gDNA or eDNA sample was treated with DNase I of different concentrations (1.45, 1.7, 2.5, and 3.0 mg/ml) (Sigma-Aldrich, DN25, USA), RNase A (0.90 mg/ml) (Sigma-Aldrich, R6513, USA), and proteinase K (0.85 mg/ml) (Sigma-Aldrich, P2308, USA) for 1 h at 37°C. Then, agarose gel electrophoresis (1.5%) was applied for 45 min at 120 V, and the products were visualized with the Vilber Lourmat Quantum ST4 Gel Documentation System. The negative controls were contained in the samples not treated by the enzymes.

Treatment of mature biofilms with DNase I. This assay was conducted as defined before by Grande et al. (2010) with a few modifications. First, TSB and bacteria culture was added to a 96-well polystyrene microtiter plate and incubated at 65°C for 40 h. The plates were then depleted and washed two times with physiological saline. The biofilms were treated with 100 μl of DNase I (Sigma-Aldrich, DN25, 100 μg/ml) for 2, 4, 8, and 12 h at 37°C. The wells were cleaned and air-dried. Finally, the CV staining assay was used. The biofilm samples were treated with physiological saline for positive controls.

Determination of biofilm mass on abiotic surfaces. The bead vortexing method was applied with some modifications for the cell viability assay on various material surfaces (Giaouris and Nychas 2006). The materials were stainless steel (grade 316L), polypropylene, polystyrene, polyvinyl chloride, polycarbonate coupons (R: 14 mm), and glass slides (26 mm × 20 mm × 1 mm). For sterilization, first, the materials were treated with isopropanol (Merck, Germany) overnight and agitated with a chlorinated detergent (Johnson & Johnson, Philippines) for 30 min. Then, the materials were washed with deionized water, air-dried, and autoclaved. The sterile materials were placed into 6-well polystyrene microtiter plates containing TSB and bacteria culture, and then the plates were incubated for 48 h. After this period, the materials were removed with sterile forceps and rinsed with 4.5 ml of physiological saline to purge planktonic cells. The material's surfaces were scratched. The materials and biofilm samples were taken to the tubes containing only glass beads and were vortexed for ~ 2 min. The drop plate method was applied to calculate the number of viable cells in MI (medium) agar plates (Herigstad et al. 2001). The results were calculated as colony-forming units per unit area (cfu/cm^2) and were log-transformed (log cfu/cm^2). Negative controls were surfaces in the only TSB.

Prevention of biofilm formation using sanitation agents on polystyrene surface. Fifteen agents were used for this assay. The agents were alkaline protease (AP; Sigma P-4860, USA), protease (Sigma P-3111, USA), subtilisin (Sigma P-5380, USA), trypsin (Sigma T-2600000, USA), sodium dodecyl sulfate (SDS; Sigma L-5750, USA), α-amylase (Sigma A-4551, USA), cellulose (Sigma C-1184, USA), sodium metaperiodate (SM; Sigma 71859, USA), lysozyme (Sigma L-7651, USA), trichloroacetic acid (TCA; Sigma 27242, USA), nisin (Sigma N-5764, USA), potassium monopersulfate (PM; Sigma 228036, USA) and sodium thiosulfate (ST; Sigma 72049, USA) combination, furanone (Sigma 283754, USA), and triclosan (Sigma LRAA-1072, USA). Bacterial culture (5 μl) and TSB (95 μl) were added in 96-well polystyrene microtiter plates. After incubation, the wells were emptied and rinsed with physiological saline. The wells were treated with 15 different sanitation agents (100 μl) under suitable conditions with some modifications (Table I). Again, the wells were emptied and washed. Finally, the CV staining assay was applied to the wells. Wells containing only the suitable solvent without sanitation agents served as the positive control. The results were calculated using the formula of Pitts et al. (2003).

Statistical analysis. All the experiments were conducted in three replicates on three independent days, and the means and the standard deviations were calculated. In the evaluation of the results obtained with the SPSS 17.0 statistical program (SPSS Inc., USA), one-way analysis of variance (ANOVA) was used to assess the difference between the averages of the values, and

Table I
Treatment of biofilms with sanitation agents.

Agent (Concentration)	Temperature – Time	References
AP (0.16 U/g)	37°C – 60 min	Parkar et al. 2004
Protease 0.16 U/g)	37°C – 60 min	Parkar et al. 2004
Subtilisin (1%)	37°C – 30 min	Parkar et al. 2004
Trypsin (3%)	37°C – 3 h	Parkar et al. 2003
SDS (3%)	100°C – 10 min	Parkar et al. 2003
α-Amylase (1%)	37°C – 30 min	Parkar et al. 2004
Cellulase (1.66%)	37°C – 30 min	Parkar et al. 2004
SM (100 mM)	22°C – 60 min	Parkar et al. 2003
Lysozyme (2%)	37°C – 60 min	Parkar et al. 2003
TCA (10%)	100°C – 15 min	Parkar et al. 2003
Nisin (2 mg/ml)	37°C – 24 h	Parkar et al. 2003
PM (2 mg/ml)	22°C – 30 min	Parkar et al. 2003
ST (10 mg/ml)	22°C – 5 min	Parkar et al. 2003
2(5H)-Furanone (1 mg/ml)	22°C – 60 min	Ponnusamy et al. 2010
Triclosan (2 mg/ml)	22°C – 60 min	Tabak et al. 2007

Tukey and Dunnett's tests were applied to compare each group in pairs. Probability levels of $p < 0.05$ were considered statistically significant.

Results

Effect of environmental conditions on the bacterial growth. The optimal planktonic and biofilm growth conditions of the D413 strain were determined at 65°C, pH 7.0, and 0% NaCl. Those of the E134 strain were similar, except that the planktonic and biofilm growth conditions' optimal pH were 8.5 and 9.0, respectively. Both bacteria did not need salt to grow and form a biofilm. Moreover, these isolates achieved optimal growth at 65°C. It was observed that these bacteria had neutral or alkaline environment requirements for their growth.

Determination of the molecular weight of gDNA and eDNA. The presence of eDNA was confirmed for the first time with electrophoresis and spectrophotometric DNA measurements for the D413 and E134 isolates. The molecular weight of gDNA was calculated as 26.1 kb and 29.8 kb for D413 and E134, respectively. Moreover, the molecular weight of eDNA was calculated as 18.2 kb and 21.4 kb for D413 and E134, respectively. It was shown that the molecular weight of the gDNA was larger than the molecular weight of the eDNA in both bacteria. In addition, the molecular weight of gDNA and eDNA of E134 was higher than in the D413 strain. Thus, the presence of eDNA in the biofilm matrix was indicated by electrophoresis and spectrophotometry.

Treatment of partially purified gDNA and eDNA with enzymes. gDNA and eDNA were partially purified in this study. The results indicated that eDNA was interestingly not affected by any of the enzymes. The enzymes could not reach the target eDNA. Furthermore, the eDNA resistance may result from the conserved folded structure of eDNA after purification. Another assumption is that the eDNA might be protected by other EPS and/or EVs structures. However, the gDNA was degraded by only DNase I. It seemed that the eDNA of the D413 and E134 isolates had resistance to DNase I (1.45 mg/ml) when partially purified (Fig. 1A). To confirm

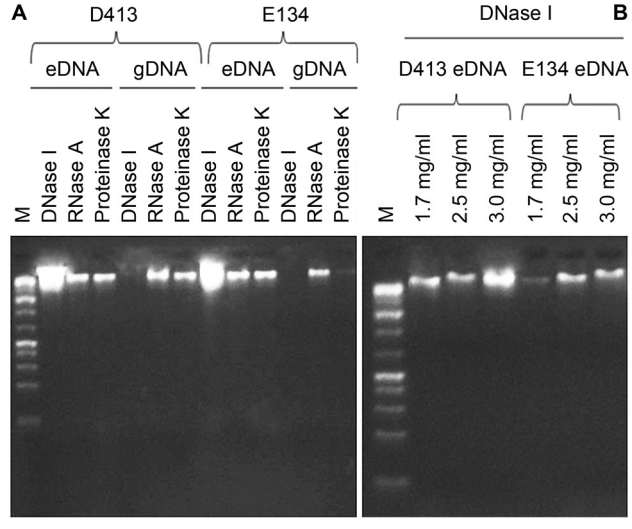

Fig. 1. Agarose gel electrophoresis photographs displaying differences between the gDNA and eDNA of D413 and E134. M, Marker (Fermentas Gene Ruler 1 kb Plus DNA Ladder, 75–20.000 bp) (A) DNase I (1.45 mg/ml), RNase A (0.90 mg/ml) and proteinase K (0.85 mg/ml) treatment of both the gDNA and eDNA; (B) Different DNase I concentrations on eDNA (1.7, 2.5 and 3.0 mg/ml).

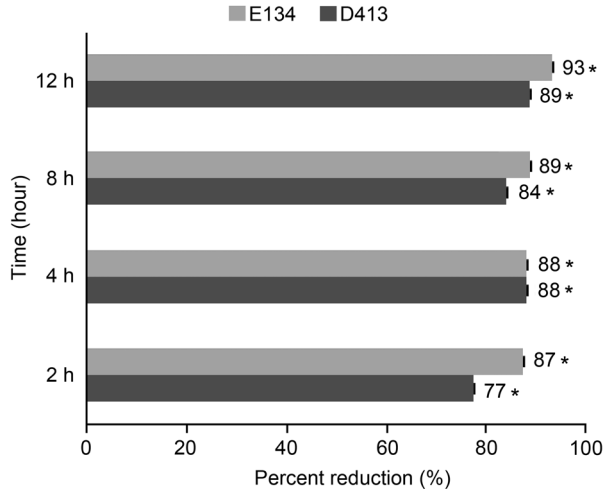

Fig. 2. Treatment of mature biofilms with DNase I. $P < 0.05$* for comparisons of data obtained in the absence and in the presence of DNase I (Dunnett's test).

the resistance, the eDNA was treated with higher concentrations of 1.7, 2.5, and 3.0 mg/ml of DNase I. However, the result remained the same (Fig. 1B).

Treatment of mature biofilms with DNase I. In this assay, partially unpurified eDNA (40 h-old mature biofilms) was treated with DNase I for 2, 4, 8, and 12 h in polystyrene plates. The mature biofilms were scattered for 2 h and 12 h of treatment with biomass removal of 77–89%, and 87–93% for D413 and E134 bacteria, respectively. It was observed that biomass removal increased up to 12 h when eDNA was partially unpurified ($p < 0.05$*) (Fig. 2).

Determination of biofilm mass on abiotic surfaces. For the assay, both scraping and bead vortexing were applied to the adherence substrate. Six abiotic surfaces were compared in terms of viable cell counts with the plate counting method. According to the results, the D413 and E134 cells could attach to all material surfaces. The viable cell numbers ranged from 3.91 to 5.12 log cfu/cm^2 and 2.25 to 4.70 log cfu/cm^2, respectively. Polystyrene (4.70 log cfu/cm^2) and glass (5.12 log cfu/cm^2) were determined to be the most effective materials for biofilm formation by D413 and E134 isolates, respectively. The relationship between each material was statistically significant ($p < 0.05$*) (Fig. 3 and 4).

Prevention of biofilm formation using sanitation agents. Fifteen different sanitation agents were used to test for the control of bacterial biofilm. The best results were provided by nisin for D413 (80%) and α-amylase for E134 (98%). This work showed that nisin, furanone, α-amylase, AP, subtilisin, SDS, TCA, lysozyme, protease, and cellulase provided over 30% biofilm removal in both bacterial species ($p < 0.05$*). Furthermore, the combination of PM and ST did not affect biofilm removal of both bacteria ($p > 0.05$) (Fig. 5).

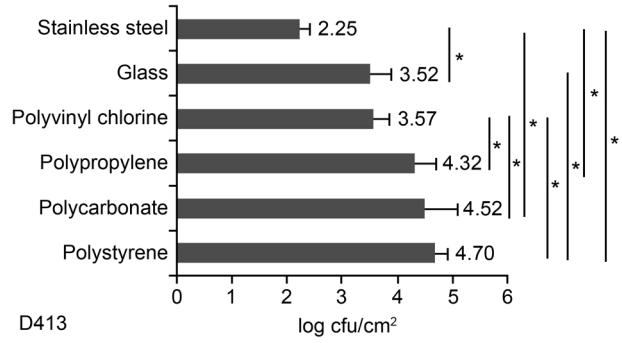

Fig. 3. The viable cell counts of D413 biofilms formed on surfaces (Tukey test; $p < 0.05$*).

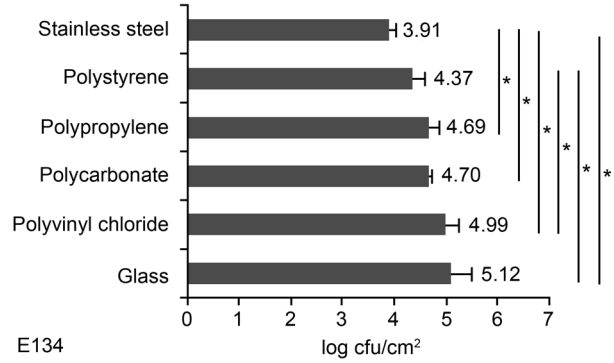

Fig. 4. The viable cell counts of E134 biofilms formed on surfaces (Tukey test; $p < 0.05$*).

Discussion

The temperature range of growth for *Geobacillus* species is 37–75°C, with the optimal temperature being from 55 to 65°C (Wells-Bennik et al. 2019). The optimal temperature for growth of the D413 and E134 isolates was 65°C. Interestingly, the necessary optimal conditions for biofilm growth of these isolates were determined to have similar values. As a result, both bacteria did not need salt to form a biofilm. The increase in the concentration of salt unfavorably affected the formation of biofilm for these isolates. In addition, these bacteria did grow in a neutral or alkaline environment. This could be explained by the intense relationship between the bacterial adherence conditions and the optimal metabolic activity (Elhariry et al. 2012).

Exopolysaccharide, proteinaceous polymers, lipids, and eDNA may be important biofilm matrix components (Allesen-Holm et al. 2006; Soler-Arango et al. 2019). Many microorganisms release eDNA within their biofilm matrix. Moreover, eDNA was reported as a component of the EPS matrix of numerous Gram-negative and Gram-positive bacteria (Ibáñez de Aldecoa et al. 2017; Ramirez et al. 2019). However, as far as it is known, there is no information about the eDNA of *Geobacillus* strain. Using electrophoresis and spectrophotometric methods, the presence of eDNA

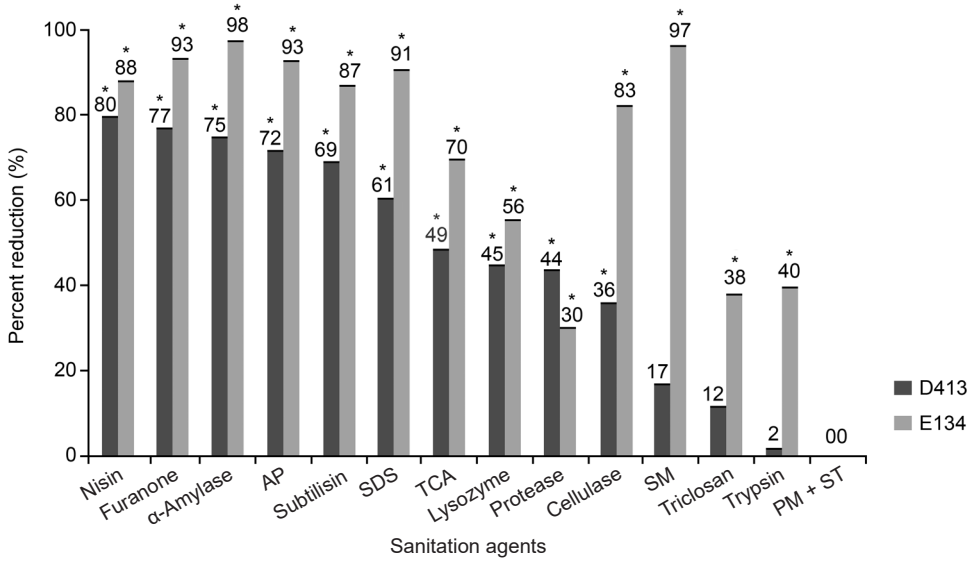

Fig. 5. The effects of sanitation agents on the biofilm of D413 and E134 isolates (Dunnett's test; $p < 0.05$*).

in D413 and E134 isolates was confirmed (Fig. 1). As a result, the gDNA's molecular weights for D413 and E134 were found as 26.1 kb, and 29.8 kb, respectively. The molecular weights of the eDNA for D413 and E134 were found as 18.2 kb, and 21.4 kb, respectively. The molecular weight of the gDNA was larger than the molecular weight of the eDNA for both bacteria.

Potential target sites of Gram-positive bacteria to antimicrobials are the cell wall, the cytoplasmic membrane, functional and structural proteins, DNA, RNA, and other cytosolic components (Bridier et al. 2011). In this study, eDNA in the biofilm matrix was not affected by DNase I, RNase A, and proteinase K enzymes. However, the gDNA was degraded only by DNase I. It seemed that the eDNA of the D413 and E134 had resistance to DNase I when purified (Fig. 1). Böckelmann et al. (2006) reported that agarose gel electrophoresis of purified microfilaments of strain F8 resulted in a distinct band of large size (more than 29 kb) and the band of the eDNA of the strain exactly disappeared after treatment with DNase I but remained stable after treatment with RNase A and proteinase K. Dengler et al. (2015) indicated that *Staphylococcus aureus* biofilm became less sensitive to proteinase K. In contrast, Nguyen and Burrows (2014) determined that proteinase K reduced biofilm formation of *Listeria monocytogenes*.

Qin et al. (2007) showed that DNase I severely decreased the biofilm formation of *Staphylococcus epidermidis*. In another study, Izano et al. (2008) reported that DNase I prevented biofilm formation of *S. aureus* and *S. epidermidis*. DNase I began to dissolve biofilms of *Bacillus subtilis* after 3 h, whereas the biofilms dissolved at the slight degree at the 24 and 48 h (Peng et al. 2020). In addition, Peng et al. (2020) reported that young biofilms were easily disturbed by DNase I, whereas the latter was not effective against aged biofilms. On the contrary, this study determined that aged biofilms of *Geobacillus* isolates were markedly affected by DNase I. As a result, DNase I reduced mature biofilms (40 h) of D413 and E134 by 77–89% and 87–93% (2–12 h), respectively ($p < 0.05$*) (Fig. 2).

Thermophilic bacteria can attach to stainless steel coupons and support biofilms' development (Jindal et al. 2016; Gupta and Anand, 2018). In this paper, six abiotic surfaces were compared in terms of viable cell counts within biofilms. D413 and E134 were observed to adhere to all surfaces. The viable cell numbers ranged from 3.91 to 5.12 log cfu/cm^2 and 2.25 to 4.70 log cfu/cm^2, respectively. Polystyrene surface (4.70 log cfu/cm^2) and glass surface (5.12 log cfu/cm^2) were determined to be the most effective surface for biofilm formation of D413 and E134 isolates, respectively ($p < 0.05$*) (Fig. 3 and 4). Karaca et al. (2019) reported that *Geobacillus vulcanii* DSM 13174^T produced the most abundant biofilm on glass and polystyrene surfaces at 65°C.

Biofilm dispersal can be provided by the disruption of the polysaccharide matrix, proteins, and eDNA. To remove irreversibly attached cells, the implementation of a powerful shear force as scrubbing, scraping or chemical breaking of the adherence forces through the applications of enzymes, sanitizers, and heat is required (Elhariry et al. 2012). It was determined that various sanitation agents could help to reduce the number of D413 and E134 cells on the polystyrene surface (Fig. 5). The best results were provided by nisin for D413 (80%) and α-amylase for E134 (98%) ($p < 0.05$*). Nisin is active upon the cytoplasmic membrane of Gram-positive bacteria, including bacterial spores, and it has been widely used as a food protective for many years

(Delves-Broughton et al. 1992; Boziaris and Adams 1999; Rojo-Bezares et al. 2007). Furthermore, nisin exhibits antimicrobial activity by binding to the pyrophosphate moiety of lipid II. Nisin also inhibited biofilm formation of *S. aureus* (Angelopoulou et al. 2020). In another study, nisin (1 mg/ml) killed 100% of *Bacillus flavothermus* B12-C^{m} biofilm cells (Parkar et al. 2003). Fleming et al. (2017) indicated that treatments of *S. aureus* and *Pseudomonas aeruginosa* biofilms with α-amylase and cellulase resulted in an important decrease in the biofilm biomass. In this study, α-amylase (98%) and SM (97%), which are glycoside hydrolases that break down polysaccharides, affected the E134 strain biofilm. Degradation of quorum sensing (QS) signals could be considered a promising approach in biofilm control (Algburi et al. 2017). The results of this study showed that furanone was very effective for removing biofilms of D413 (77%) and E134 (93%) ($p < 0.05^{*}$) strains. 2(5H)-Furanone acts as a potential quorum-inhibition agent in a biofilm community and could displace the AHL signals from the LuxR protein. Furanones have been commonly used to remove biofilm from medical catheters and diverse other substrates (Ponnusamy et al. 2010). In this study, it was observed that both protein-degrading agents and polysaccharide-degrading agents were effective for the biofilm control of *Geobacillus*. Lequette et al. (2010) found that proteases were more efficient than polysaccharides for the removal of *Bacillus* spp. biofilms while polysaccharide-degrading enzymes were more efficient for control of *Pseudomonas fluorescens* biofilms. However, the combination of PM and ST had no inhibitory effect on biofilm formation of both bacteria ($p > 0.05$) (Fig. 5).

In conclusion, this paper showed that DNase I degraded the eDNA of *Geobacillus* genus bacteria. The importance of eDNA for mature biofilm stability after the DNase I application was demonstrated. Besides, sanitation agents like α-amylase, nisin, and furanone significantly impacted these bacteria's biofilm formation. This work suggests that sanitation agents could be a solution to control biofilm structures of thermophilic bacilli. Biofilm control can be determined by a combination of these sanitation agents in future studies. It seemed that *Geobacillus* strains could form biofilms on stainless steel, glass, and plastic surfaces. In addition, this work suggest that biofilm control could be improved by using new sanitation strategies on these surfaces.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Algburi A, Comito N, Kashtanov D, Dicks LM, Chikindas ML. Control of biofilm formation: antibiotics and beyond. App Environ Microbiol. 2017 Feb; 83(3):e02508–16. https://doi.org/10.1128/AEM.02508-16

Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, Molin S, Givskov M, Tolker-Nielsen T. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol Microbiol. 2006 Nov; 59(4):1114–1128. https://doi.org/10.1111/j.1365-2958.2005.05008.x

Angelopoulou A, Field D, Pérez-Ibarreche M, Warda AK, Hill C, Ross RP. Vancomycin and nisin A are effective against biofilms of multi-drug resistant *Staphylococcus aureus* isolates from human milk. Plos One. 2020 May; 15(5):e0233284. https://doi.org/10.1371/journal.pone.0233284

Boziaris IS, Adams MR. Effect of chelators and nisin produced in situ on inhibition and inactivation of Gram negatives. Int J Food Microbiol. 1999 Jul; 53(2–3):105–113. https://doi.org/10.1016/S0168-1605(99)00139-7

Böckelmann U, Janke A, Kuhn R, Neu TR, Wecke J, Lawrence JR, Szewzyk U. Bacterial extracellular DNA forming a defined network-like structure. FEMS Microbiol Lett. 2006 Sep; 262(1):31–38. https://doi.org/10.1111/j.1574-6968.2006.00361.x

Bremer P, Seale B, Flint S, Palmer J. Biofilms in dairy processing. In: Fratamico PM, Annous BA, Gunther IV NW, editors. Biofilms in the food and beverage industries. Sawston (UK): Woodhead Publishing. 2009; p. 396–431. https://doi.org/10.1533/9781845697167.4.396

Bridier A, Briandet R, Thomas V, Dubois-Brissonnet F. Resistance of bacterial biofilms to disinfectants: a review. Biofouling. 2011 Oct; 27(9):1017–1032. https://doi.org/10.1080/08927014.2011.626899

Burgess SA, Brooks JD, Rakonjac J, Walker KM, Flint SH. The formation of spores in biofilms of *Anoxybacillus flavithermus*. J Appl Microbiol. 2009 Feb; 107(3):1012–1018. https://doi.org/10.1111/j.1365-2672.2009.04282.x

Burgess SA, Flint SH, Lindsay D. Characterization of thermophilic bacilli from a milk powder processing plant. J Appl Microbiol. 2013 Oct; 116(2):350–359. https://doi.org/10.1111/jam.12366

Cihan AC, Ozcan B, Tekin N, Cokmus C. Phylogenetic diversity of isolates belonging to genera *Geobacillus* and *Aeribacillus* isolated from different geothermal regions of Turkey. World J Microb Biot. 2011 Apr;27(11):2683. https://doi.org/10.1007/s11274-011-0742-2

Cihan AC, Karaca B, Ozel BP, Kilic T. Determination of the biofilm production capacities and characteristics of members belonging to Bacillaceae family. World J Microb Biot. 2017 May;33(6):118. https://doi.org/10.1007/s11274-017-2271-0

Delves-Broughton J, Williams GC, Wilkinson S. The use of the bacteriocin, nisin, as a preservative in pasteurized liquid whole egg. Lett Appl Microbiol. 1992 Oct;15(4), 133–136. https://doi.org/10.1111/j.1472-765X.1992.tb00746.x

Dengler V, Foulston L, DeFrancesco AS, Losick R. An electrostatic net model for the role of extracellular DNA in biofilm formation by *Staphylococcus aureus*. J Bacteriol. 2015 Sep;197(24):3779–3787. https://doi.org/10.1128/JB.00726-15

Eijlander RT, van Hekezen R, Bienvenue A, Girard V, Hoornstra E, Johnson NB, Meyer R, Wagendorp A, Walker DC, Wells-Bennik MHJ. Spores in dairy-new insights in detection, enumeration and risk assessment. Int J Dairy Technol. 2019 May;72(2):303–315. https://doi.org/10.1111/1471-0307.12586

Elhariry H, Gherbawy Y, El-Deeb B, Altalhi A. Molecular identification and biofilm-forming ability of culturable aquatic bacteria in microbial biofilms formed in drinking water distribution networks. Geomicrobiol J. 2012 May;29(6):561–569. https://doi.org/10.1080/01490451.2011.596254

Fleming D, Chahin L, Rumbaugh K. Glycoside hydrolases degrade polymicrobial bacterial biofilms in wounds. Antimicrob Agents Ch. 2017 Nov; 61(2):e01998-16. https://doi.org/10.1128/AAC.01998-16

Garrett TR, Bhakoo M, Zhang Z. Bacterial adhesion and biofilms on surfaces. Prog Nat Sci. 2008 Sep;18(9):1049–1056. https://doi.org/10.1016/j.pnsc.2008.04.001

Giaouris ED, Nychas GJE. The adherence of *Salmonella Enteritidis* PT4 to stainless steel: The importance of the air-liquid interface and nutrient availability. Food Microbiol. 2006 Apr;23:747–752. https://doi.org/10.1016/j.fm.2006.02.006

Gopal N, Hill C, Ross PR, Beresford TP, Fenelon MA, Cotter PD. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. Front Microbiol. 2015 Dec;6:1418. https://doi.org/10.3389/fmicb.2015.01418

González-Rivas F, Ripolles-Avila C, Fontecha-Umaña F, Ríos-Castillo AG, Rodríguez-Jerez JJ. Biofilms in the spotlight: Detection, quantification, and removal methods. Compr Rev Food Sci Food Saf. 2018 Jun;17(5):1261–1276. https://doi.org/10.1111/1541-4337.12378

Grande R, Di Giulio M, Bessa LJ, Di Campli E, Baffoni M, Guarnieri S, Cellini L. Extracellular DNA in *Helicobacter pylori* biofilm: a backstairs rumour. J Appl Microbiol. 2010 Nov;110:490–498. https://doi.org/10.1111/j.1365-2672.2010.04911.x

Gupta S, Anand S. Induction of pitting corrosion on stainless steel (grades 304 and 316) used in dairy industry by biofilms of common sporeformers. Int J Dairy Technol. 2018 May;71(2):519–531. https://doi.org/10.1111/1471-0307.12444

Herigstad B, Hamilton M, Heersink J. How to optimize the drop plate method for enumerating bacteria. J Microbiol Meth. 2001 Mar;44:121–129. https://doi.org/10.1016/s0167-7012(00)00241-4

Ibáñez de Aldecoa AL, Zafra O, González-Pastor JE. Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. Front Microbiol. 2017 Jul;8:1390. https://doi.org/10.3389/fmicb.2017.01390

Izano EA, Amarante MA, Kher WB, Kaplan JB. Differential roles of poly-N acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Appl Environ Microbiol. 2008 Jan;74(2):470–476. https://doi.org/10.1128/AEM.02073-07

Jindal S, Anand S, Huang K, Goddard J, Metzger L, Amamcharla J. Evaluation of modified stainless steel surfaces targeted to reduce biofilm formation by common milk sporeformers. J Dairy Sci. 2016 Sep;99(12):9502–9513. https://doi.org/10.3168/jds.2016-11395

Karaca B, Buzrul S, Coleri Cihan A. *Anoxybacillus* and *Geobacillus* biofilms in the dairy industry: effects of surface material, incubation temperature and milk type. Biofouling. 2019 Jul;35(5):551–560. https://doi.org/10.1080/08927014.2019.1628221

Lequette Y, Boels G, Clarisse M, Faille C. Using enzymes to remove biofilms of bacterial isolates sampled in the food-industry. Biofouling. 2010 May; 26(4): 421–431. https://doi.org/10.1080/08927011003699535

Lindsay D, Flint S. Biofilm formation by spore-forming bacteria in food processing environments. In: Fratamico PM, Annous BA, Gunther IV NW, editors. Biofilms in the food and beverage industries. Sawston (UK): Woodhead Publishing. 2009; p. 270–299. https://doi.org/10.1533/9781845697167.2.270

Mandic-Mulec I, Stefanic P, van Elsas JD. Ecology of *Bacillaceae*. Microbiol Spect. 2015 Mar;3(2):1–24. https://doi.org/10.1128/microbiolspec.TBS-0017-2013

Mazaheri T, Ripolles-Avila C, Hascoët AS, Rodríguez-Jerez JJ. Effect of an enzymatic treatment on the removal of mature *Listeria monocytogenes* biofilms: A quantitative and qualitative study. Food Control, 2020 Mar;107266. https://doi.org/10.1016/j.foodcont.2020.107266

Meireles A, Borges A, Giaouris E, Simões M. The current knowledge on the application of anti-biofilm enzymes in the food industry. Food Res Int. 2016 Jun; 86:140–146. https://doi.org/10.1016/ j.foodres.2016.06.006

Nguyen UT, Burrows LL. DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms. Int J Food Microbiol. 2014 Jul;187:26–32. https://doi.org/10. 1016/j.ijfoodmicro.2014.06.025

Parkar SG, Flint SH, Brooks JD. Physiology of biofilms of thermophilic bacilli – potential consequences for cleaning. J Ind Microbiol Biot. 2003 Aug;30: 553–560. https://doi.org/10.1007/s10295-003-0081-x

Parkar SG, Flint SH., Brooks JD. Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. J Appl Microbiol. 2004 Nov; 96:110–116. https://doi.org/10.1046/j.1365-2672.2003.02136.x

Peng N, Cai P, Mortimer M, Wu Y, Gao C, Huang Q. The exopolysaccharide-eDNA interaction modulates 3D architecture of *Bacillus subtilis* biofilm. BMC Microbiol. 2020 May;20:1–12. https://doi.org/10.1186/s12866-020-01789-5

Pitts B, Hamilton MA, Zelver N, Stewart PS. A microtiter-plate screening method for biofilm disinfection and removal. J Microbiol Meth. 2003 Aug; 54:269–276. https://doi.org/10.1016/S0167-7012(03)00034-4

Ponnusamy K, Paul D, Kim YS, Kweon JH. 2(5H)-Furanone: a prospective strategy for biofouling-control in membrane biofilm bacteria by quorum sensing inhibition. Braz J Microbiol. 2010 Mar;41:227–234. https://doi.org/10.1590/S1517-83822010000100032

Ramirez T, Shrestha A, Kishen A. Inflammatory potential of monospecies biofilm matrix components. In Endod J 2019 Feb;52(7): 1020–1027. https://doi.org/10.1111/iej.13093

Rojo-Bezares B, Sáenz Y, Zarazaga M, Torres C, Ruiz-Larrea F. Antimicrobial activity of nisin against *Oenococcus oeni* and other wine bacteria. Int. J Food Microbiol. 2007 May;116(1):32–36. https://doi.org/10.1016/j.ijfoodmicro.2006.12.020

Rumbaugh KP, Sauer K. Biofilm dispersion. Nat Rev Microbiol. 2020 Jun;18:571–586. https://doi.org/10.1038/s41579-020-0385-0

Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, Qu D. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology. 2007 Jul;153(7):2083–2092. https://doi.org/10.1099/mic.0.2007/006031-0

Ripolles-Avila C, Ríos-Castillo AG, Fontecha-Umaña F, Rodríguez-Jerez JJ. Removal of *Salmonella enterica* serovar Typhimurium and *Cronobacter sakazakii* biofilms from food contact surfaces through enzymatic catalysis. J Food Safety. 2019 Dec;40(2):e12755. https://doi.org/10.1111/jfs.12755

Ripolles-Avila C, Ramos-Rubio M, Hascoët AS, Castillo M, Rodríguez-Jerez JJ. New approach for the removal of mature biofilms formed by wild strains of *Listeria monocytogenes* isolated from food contact surfaces in an Iberian pig processing plant. Int J Food Microbiol. 2020 Mar;108595. https://doi.org/10.1016/j.ijfoodmicro.2020.108595

Scott SA, Brooks JD, Rakonjac J, Walker KM, Flint SH. The formation of thermophilic spores during the manufacture of whole milk powder. Int J Dairy Technol. 2007 May;60(2):109–117. https://doi.org/10.1111/j.1471-0307.2007.00309.x

Shemesh M, Ostrov I. Role of *Bacillus* species in biofilm persistence and emerging antibiofilm strategies in the dairy industry. J Sci Food Agric. 2020 Jan; 100(6):2327–2336. https://doi.org/10.1002/jsfa.10285

Soler-Arango J, Figoli C, Muraca G, Bosch A, Brelles-Mariño G. The *Pseudomonas aeruginosa* biofilm matrix and cells are drastically impacted by gas discharge plasma treatment: A comprehensive model explaining plasma-mediated biofilm eradication. PloS One. 2019 Jun;14(6):e0216817. https://doi.org/10.1371/journal.pone.0216817

Stepanović S, Vuković D, Dakić I, Savić B, Švabić-Vlahović M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Meth. 2000 Apr;40:175–179. https://doi.org/10.1016/ s0167-7012(00)00122-6

Sung MH, Kim H, Bae JW, Rhee SK, Jeon CO, Kim K, Kim JJ, Hong SP, Lee SG, Yoon JH, Park YH, Baek DH. *Geobacillus toebii* sp. nov., a novel thermophilic bacterium isolated from hay compost. Int J Syst Evol Micr. 2002 Jun;52(6):2251–2255. https://doi.org/10.1099/00207713-52-6-2251

Tabak M, Scher K, Hartog E, Romling U, Matthews KR, Chikindas ML, Yaron S. Effect of triclosan on *Salmonella typhimurium* at different growth stages and in biofilms. FEMS Microbiol Lett. 2007 Dec;267:200–206. https://doi.org/10.1111/j.1574-6968.2006.00547.x

Wells-Bennik MH, Janssen PW, Klaus V, Yang C, Zwietering MH, Den Besten HM. Heat resistance of spores of 18 strains of *Geobacillus stearothermophilus* and impact of culturing conditions. Int J Food Microbiol. 2019 Nov;291:161–172. https://doi.org/10.1016/j.ijfoodmicro.2018.11.005

Wilson K. Preparation of genomic DNA from bacteria. Curr Protoc Mol Biol. 2001 Nov;56:2–4. https://doi.org/10.1002/0471142727.mb0204s56

Woodward MJ, Sojka M, Sprigings KA, Humphrey TJ. The role of SEF14 and SEF17 fimbriae in the adherence of *Salmonella enterica* serotype Enteritidis to inanimate surfaces. J Med Microbiol. 2000 May;49:481–487. https://doi.org/10.1099/0022-1317-49-5-48