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Influence of milk proteins on the adhesion and formation of *Bacillus sporothermodurans* biofilms: Implications for dairy industrial processing

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ABSTRACT

Bacillus sporothermodurans is a producer of highly heat-resistant spores, which is a problem for the dairy industry worldwide. In this work, we studied the attachment and biofilm formation on stainless steel surfaces in contact with milk proteins (casein and whey). The results of the biofilm formation were obtained by the plate count method. In conjunction, biofilms (niches and cell sizes) were measured by scanning electron microscopy (SEM) on the 1st, 5th and 10th day. X-ray photoelectron spectroscopy (XPS) was a complementary technique that probed the conditioning of the substrate surface after 24 h. The milk proteins affected the total bacterial count in the samples, and also influenced the biofilm architecture. Sessile cell counts varied from 4.3 log CFU/cm² in the attachment stage to up to 10.2 log CFU/cm² in the matured biofilm stage. The spore counts varied from $<1.4 \pm 0.02$ (1st day) to $3.7 \log \pm 1.1$ log spores/cm² (10th day). The control group at the biofilm maturation stage (5th day) had higher niches than at the cell fixation (1st day) and was different from the other groups that showed active dispersion in the presence of milk proteins. The same occurred on the 10th day for the control group. Circular patterns in the biofilm with casein, passive dispersal and elongated cells were also observed. Enzyme treatment and disinfectants may be used to remove or reduce biofilm formation.

1. Introduction

The definition of biofilm in the dairy industry is similar to that of the industrial and clinical environments. Biofilms are structured bacterial communities embedded in a self-produced polymeric matrix that confers emergent properties to the species (Costerton et al., 1999; Stoodley et al., 2002). However, in the dairy industry, the milk composition can affect bacterial adhesion (Flint et al., 2015; Speers & Gilmour, 1985). Milk is a biochemical complex rich in proteins, fat globules, lactose, minerals, vitamins, with a neutral pH (6.4–6.8), high water activity (aw: 0.99), and oxidation-reduction potential (Eh: > 0.3 mV), suitable for providing growth to aerobic microorganisms (Flint et al., 2015; Hassan & Frank, 2011; Huppertz & Kelly, 2009).

The presence of milk residues from the production process conditions the stainless steel (SS) surfaces for biofilm formation, as these nutrients are adsorbed onto the surface. Therefore, biofilm formation on surfaces directly in contact with milk, such as heat exchangers, evaporators,

tubes, and reservoirs is common (Anand et al., 2014; Flint et al., 2015). Some bacterial species (*Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Alcaligenes*, and *Moraxella*-like) can fix onto glass, rubber, and SS surfaces and they show high adherence with lactose and non-casein proteins due to the synthesis of an essential polymer for cell adhesion (Flint et al., 2015; Speers & Gilmour, 1985). On the other hand, the adhesion of *Staphylococcus aureus*, *Listeria monocytogenes* and *Serratia marcescens* onto the SS surface is reduced in the presence of skimmed milk, as well as individual milk proteins (Barnes et al., 1999). The κ -casein has also shown an inhibitory effect on biofilm formation and virulence of *L. monocytogenes* (Yun et al., 2014). The peptide-based coating has also been tested to modify the physical properties of SS surfaces and resulted in the reduction of *Bacillus licheniformis* and *Pseudomonas aeruginosa* (Friedlander et al., 2019). In skimmed milk, high biofilm dispersal can occur in mono or multispecies of *Bacillus cereus*, *L. monocytogenes* and *S. aureus*, except for *Enterococcus faecalis* (Alonso & Kabuki, 2019).

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Complex biological structures, such as biofilms, promote interspecies and intra-species interaction, including spore-forming bacteria that eventually contaminate the milk in dairy farms (Flint et al., 2015; Speers & Gilmour, 1985). *Bacillus sporothermodurans* (common in raw milk) may remain in ultra-high temperature (UHT) milk after inadequate heat treatment and eventually form biofilms on the surfaces of equipment in the dairy industry. However, we are not aware of studies that report the influence of milk proteins on the biofilm formation of this microorganism (Deeth & Datta, 2011; Pettersson et al., 1996; Walstra et al., 2006). This bacterium is also common in animal feed (concentrate and soybean meal), silage, filter cloths, green crops, and fodder (Logan & Vos, 2015; Scheldeman et al., 2002, 2006). The first reports in the literature for *B. sporothermodurans* isolated from UHT milk were in 1985 (Italy), 1990 (Austria) and 1995 (Germany). However, few scientific studies demonstrate the behavior of this microorganism in biofilms (Gupta & Anand, 2018; Jindal & Anand, 2018; Jindal et al., 2016; Logan & Vos, 2015; Pettersson et al., 1996).

B. sporothermodurans is Gram-positive, with optimal growth at 37 °C, 5–9 pH, and NaCl concentration of up to 5% (Logan & Vos, 2015; Pettersson et al., 1996). It is difficult to isolate and culture this microorganism from UHT milk in the laboratory, even when using a nutrient-rich culture medium such as brain heart infusion agar (BHI) (Pettersson et al., 1996). On the other hand, bacterial growth is possible in the presence of Cobalamin (vitamin B12). The development of *B. sporothermodurans* biofilms on equipment surfaces in the UHT milk processing line can be a source of contamination in the final product, which can lead to microbial growth above 10⁶ CFU/mL during storage at 30 °C for 5 days. Although this bacteria does not spoil the milk and is not pathogenic, this species has become a widespread problem in the dairy industry, due to spore heat treatment resistance, corrosion, and susceptibility to sanitizers (Jindal & Anand, 2018; Pettersson et al., 1996).

Non-pathogenic *B. sporothermodurans* is correlated with high counts of aerobic mesophilic bacteria in UHT milk. Therefore, *B. sporothermodurans* can multiply up to 10⁵ CFU/mL, while producing no sensory changes (gas production, acids, and hydrolyzed casein) during storage (Gupta & Anand, 2018; Lewis, 2014). However, it is a concern for the dairy industry as they produce exceptionally heat-resistant spores (HRS) and biofilms, which may shelter other pathogens and spoilage microorganisms (Gopal et al., 2015; Tabit & Buys, 2011; Weber et al., 2019). Moreover, biofilms induce pitting and corrosion on stainless steel, which makes the elimination of these inconvenient microorganisms even more difficult for the dairy industry (Aouadhi et al., 2016; Flint et al., 2011; Jindal et al., 2016). Biofilm formation can affect the heat transfer coefficient in heat exchangers, increasing the energy demand to maintain flow rates. In addition, production time is shortened in proportion to the time spent with cleaning and disinfection (Flint et al., 2011; Mittelman et al., 1998).

The presence of *B. sporothermodurans* is a recurrent concern in the dairy industry due to its persistence in production plants and difficulty in removal. Thus, we believe that evaluating the influence of milk proteins (casein and whey) on behavior of *B. sporothermodurans* on stainless steel at different biofilm formation stages (adhesion, maturity and dispersion) is important additional information for the prevention and inhibition of biofilms in dairy plants.

2. Material and methods

2.1. Bacterial strains and inoculum preparation

For the inoculum, we used *B. sporothermodurans* DSMZ 10599 isolated from the Italian UHT milk industry (German collection of microorganisms and cell cultures, Braunschweig, Germany), and two strains from our collection that were isolated from UHT milk. All strains were stored at –20 °C in 30% glycerol solution. Before biofilm formation, each microorganism was cultured separately into a brain heart infusion broth (Difco, Becton, Dickinson and Company, Sparks - USA),

supplemented with vitamin B12 (Sigma-Aldrich, Steinheim, Germany) (BHI-B12) for 24 h at 37 °C. Inoculum preparation for biofilm formation was performed using a pool of all three strains (2 mL each). The bacterial inocula were standardized to 1 MacFarland (~3 × 10⁸ CFU/mL) using a densitometer (model 99234, BioMérieux, Marcy l'Etoile, France). Dilutions were then done to reach a nominal inocula of approximately 10⁵ CFU/mL.

2.2. Cleaning and preparation of stainless steel surface

Stainless steel surfaces (AISI 304) utilized for the assays had dimensions of 1.0 cm × 1.0 cm, 0.1 cm thickness, and were obtained from Ecolab®/Nalco (Brazil) (Fig. 1a). Initial roughness (0.34 ± 0.03 µm) was verified using a profilometer (Dektak 150, Veeco Instruments, Tucson, USA). The homogeneity of SS surfaces was evaluated by scanning electron microscope (SEM) (Phenom, FEI Company, Hillsboro, OR, US) and the surface chemical composition was evaluated through X-ray photoelectron spectroscopy (XPS) model VSW HA-100 (VSW Scientific Instrument LTDA, England).

The SS surfaces were cleaned with acetone to remove grease from the manufacturing process and then washed by sonication (Sharp UT-204, Sharp Company, Tokyo, Japan) with a 1% NaOH solution for 30 min, followed by rinsing in sterile water and submerged per immersion in 1% nitric acid for 15 min. After, they were rinsed twice in distilled water and sterilized at 121 °C for 15 min (Antonioni & Frank, 2005). The SS surfaces were reused among repetitions of the same experiments and kept separate until use. Before biofilm formation, SS samples were imaged by optical microscopy to check for biofilm cleaning and removal, and were also visually confirmed using a microbial biodetector (BioFinder, iTram® Higiene, Spain). This product promotes an enzymatic reaction per decomposition of hydrogen peroxide, forming bubble zones and indicating a positive reaction (iTram® iTram Higiene, 2020a; Ripolles-Avila et al., 2019; Ripolles-Avila et al., 2018).

2.3. In vitro *B. sporothermodurans* biofilm formation for evaluation of milk protein effects

Four experiments were performed in independent triplicates to evaluate the effect of milk proteins on *in vitro* biofilms (Fig. 1b). The SS samples were suspended by nylon® thread and immersed in 60 mL of different media concentrations. Experiment 1 was the control assay, with BHI-B12 culture medium only. In Experiment 2, 7 g/L of whey proteins (WPC 80 standards, Arla foods - SP, Brazil) were added to the BHI-B12. In Experiment 3, we used 28 g/L of casein (Sodium caseinate, Alibra Campinas - SP, Brazil) instead, and for Experiment 4, both milk proteins were used. This preparation was also performed for the control samples, as measured by XPS (Section 2.5). The milk proteins used correspond to the average protein content per liter of milk (~3.3%), where casein concentration varies from 75% to 85%, and whey proteins from 15% to 22% (Flint et al., 2001; Huppertz & Kelly, 2009). The inoculum was diluted in the culture medium until a concentration of 10⁴ CFU/mL was reached. All experiments were kept at 35 °C (optimal growth temperature), in static conditions. The initial pH was adjusted to 7 and measured before sessile cell removal. *B. sporothermodurans* biofilm formation was observed for two SS samples, after 24 h (Day 1), 72 h (Day 3), 120 h (Day 5), 168 h (Day 7), and 240 h (Day 10).

2.4. Sessile cells and spore count of *B. sporothermodurans* biofilm

For each count, SS samples were transferred to a 10 mL sterile saline solution (0.85% NaCl wt/vol), and kept for 1 min to remove poorly adhered cells (planktonic cells). For removal of sessile cells, the SS samples were immersed in a tube containing 10 mL of a sterile saline solution and 5 g of sterile glass beads, then submerged in an ultrasonic bath (Sharp UT-204) for 5 min, and then evaluated through optical microscopy (Andrade et al., 1998; Hamilton et al., 2009). Whereas for

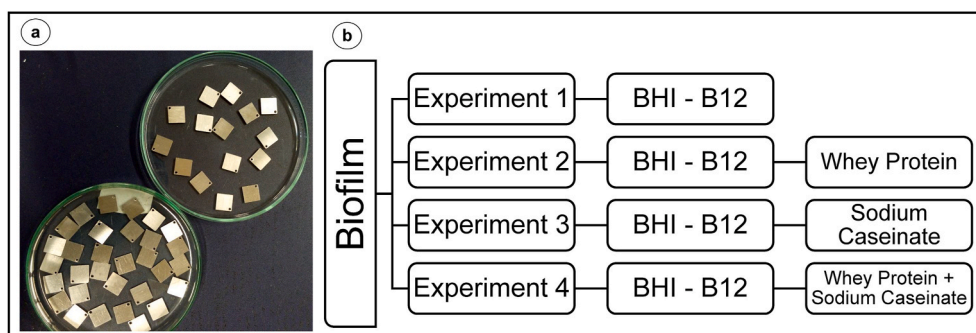


Fig. 1. Stainless steel surface and flowchart of experimental design. (a) Stainless steel surfaces (AISI 304). (b) Flowchart of experimental design for the evaluation of milk proteins effect.

spore counts, a tube containing the removed biofilm cells was placed in a water bath at 100 °C for 30 min, to eliminate vegetative cells and induce spore germination. After sessile cell removal and thermal shock, they were diluted with a saline solution (0.85% NaCl). Then, an aliquot (0.1 mL) was spread onto a BHI-B12 agar (Difco/Sigma-Aldrich) and incubated at 35 °C for 24 h. The counts were performed on days 1, 3, 5, 7, and 10. For each day, two samples were counted. The data were transformed into log CFU and calculated using the following equation:

$$\text{Log} \left(\frac{\text{CFU}}{\text{cm}^2} \right) = \left[\left(\frac{M}{V_A} \right) \times \left(\frac{V_D}{A} \right) \right] \times D \quad (1)$$

where: V_D = rinsing volume (mL); V_A = aliquot plated volume (mL); M = average colony number after incubation on plate (CFU); D = decimal dilution; and A = SS surface area (cm²) (Campos Bernardes et al., 2012; Goeres et al., 2009).

2.5. Surface analysis of conditioning film

The chemical composition of the conditioned SS surface by milk proteins was analyzed using XPS (hemispherical analyzer VSW HA-100). The measurements were performed in an ultra-high vacuum environment (UHV) with $\sim 2.10^{-8}$ mbar pressure. The X-ray source used was an aluminum anode with Al K α emission ($h\nu = 1486.6$ eV) and analyzer pass energy of 44 eV. Four SS surfaces were treated at the same culture medium concentration for 24 h, as previously described (Section 2.3), without bacterial inoculum. Before introducing the samples into the XPS UHV chamber, they were rinsed with deionized water, dried in a laminar airflow cabinet at room temperature, and stored in a desiccator. XPS data of these samples were compared with those from the clean SS surface (control).

2.6. Characterization of *B. sporothermodurans* biofilm by scanning electron microscopy (SEM)

The biofilms were observed using SEM (Phenom, FEI Company, Eindhoven, NL) on days 1, 5 and 10. The samples were prepared according to Lorite et al. (2011). The surface structures were identified in the images and the corresponding areas measured using Fiji (distribution of the ImageJ software, US National Institutes of Health, Bethesda, Maryland, USA) (Schindelin et al., 2012).

2.7. Efficacy of enzymatic treatment and disinfectants against *B. sporothermodurans* biofilm by single tube method (STM)

The Single Tube Method (ASTM E2871) was performed to evaluate the efficacy of enzymatic products and disinfectants against *B. sporothermodurans* biofilms, which were grown according to section 2.4 (Sessile cells and spore counts of *B. sporothermodurans* biofilm), in BHI-B12 for 24 h (cell attachment). The initial inoculum was 10^8 CFU/

mL. For each disinfectant, 3 untreated controls and 5 treated surfaces were used. The preparation of products (enzymatic or disinfectant) was carried out according to the manufacturer's instructions. The products were evaluated for 15 min (Biojet + EnzyJet Plus) and 30 min (Enzycip) at 50 °C (Itram®, Barcelona, Spain), and Vortexx™ and Vortexx™ ES (Ecolab®, Campinas, Brazil) for 10 min at 50 °C and 15 min at 40 °C, respectively. For Ecolab® products, the neutralizer used was DE broth (Acumedia, MI, USA), while a mixture of 30 g/L Tween 80, 3 g/L lecithin and 1 g/L L-histidine was used for iTram® products. The biofilm was removed and disaggregated via vortexing and sonication, as described in section 2.4. Then, the sessile cells were counted on BHI B12 and incubated at 35 °C. The data obtained for both control and treated surfaces (CFUs) were transformed into log densities (LD; log CFU/cm²). For all experiments, the logarithmic reduction (LR) was calculated by subtracting the mean for treated biofilm (LD_T) from the mean of untreated controls (LD_C) (ASTM, 2019; Goeres et al., 2019). This process was analyzed using SEM. In conjunction, surface chemical analyses were performed with XPS, after application of the 4 products (Biojet + EnzyJet Plus, Enzycip (Itram), Vortexx™, and Vortexx™ ES (Ecolab®)).

2.8. Statistical analysis

All statistics were calculated in OriginPro software, Version 2019b (OriginLab Corporation, Northampton, MA, USA). Data were tested for normality using Shapiro Wilk and Levene's test, with $p \leq 0.05$ for all responses. The influence of whey and caseins on biofilm formation (sessile cells and spores) were evaluated using Two-way ANOVA and Tukey's multiple comparisons test (P-values lower than 5%). For the analysis of the biofilm areas, the normally distributed samples were analyzed using one-way ANOVA and Tukey post hoc tests, whereas non-normally distributed data were analyzed using Kruskal Wallis and Dunn's tests. The level of significance was 5%.

3. Results and discussion

3.1. Surface analysis results of conditioning film and conditioned surfaces

The XPS spectra showed the presence of organic material after 24 h, which characterizes surface conditioning and favors biofilm formation. In the dairy industry, the conditioning films form when organic milk components are adsorbed onto surfaces in a fluid environment. This stage occurs in timescales of minutes to hours, modifying the physico-chemical properties of surfaces (surface free energy, hydrophobicity, and electrostatic charges) (Barnes et al., 1999; Kumar et al., 2021; Marchand et al., 2012). Fig. 2 shows the survey spectra for each experiment, as well as the control sample. In the control Experiment, a high Oxygen (O 1s) peak, resulting from surface oxides, as well as adventitious carbon (C 1s) from contamination during air exposure was found. Elements present in the chemical composition of the stainless steel alloy were also observed in the spectra, such as Fe 2p (Iron) and Cr

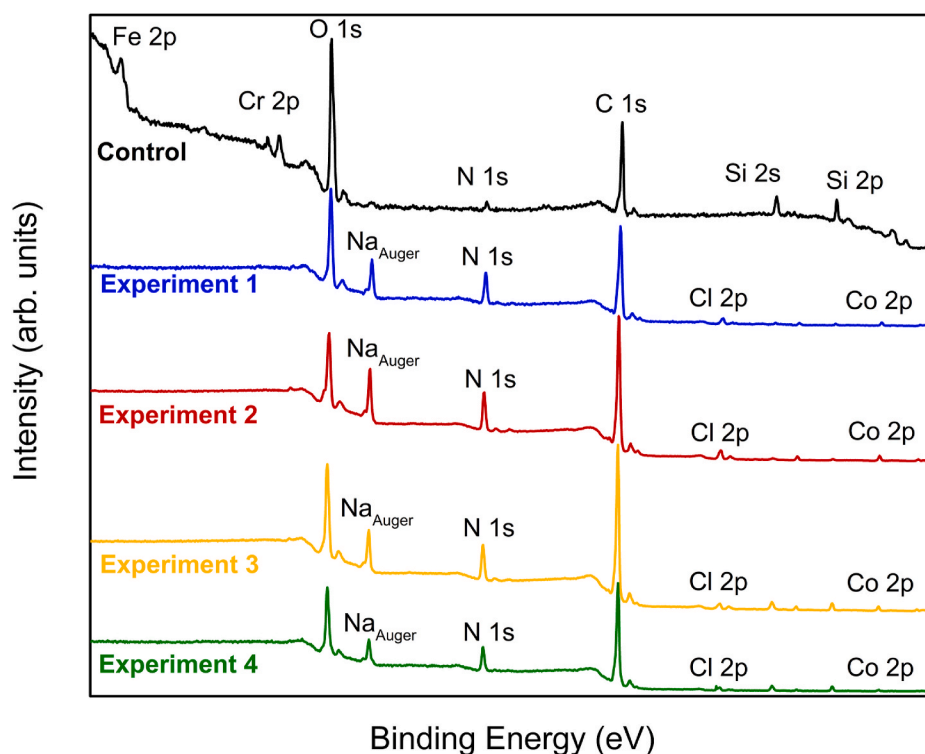


Fig. 2. XPS survey spectra for each Experiment. Control: SS clean surface; Exp. 1: BHI-B12; Exp. 2: BHI-B12-W; Exp. 3: BHI-B12-C; and Exp. 4: BHI-B12-W/C.

2p (Chromium) peaks. These results concur with previous reports (Barnes et al., 2001; Bernbom et al., 2009).

Samples obtained in Experiments 1, 2, 3, and 4 show significant changes in their corresponding spectra. The Fe 2p and Cr 2p peaks vanish due to the formation of a conditioning film, which coats the SS surface, whereas N 1s (Nitrogen), Cl 2p (Chlorine), and Co 2p (Cobalt) peaks appear. Nitrogen and carbon (C 1s) reflect the presence of organic compounds, common in the culture media and in milk protein, whereas Co is derived from vitamin B12 and Chlorine from the BHI (NaCl).

3.2. Sessile cells and spore counting

A pool of three *B. sporothermodurans* strains was used to promote synergy among the strains and, consequently, the formation of a robust biofilm. Thus, the biofilm life cycle was evaluated under the influence of

milk proteins, starting from cell attachment, maturation, dispersal, and sporulation. Therefore, the observed life cycle (10 days) considered the ability of biofilm formation in dairy plants with an absence of adequate cleaning regimes. The initial pH (1st day) was 7 for all experiments, while the final pH (10th day) was 9 for BHI-B12 (Exp. 1) and 8 for the other groups (Exp. 2, 3 and 4).

After the biofilm removal from SS surfaces and cell disaggregation, sessile cell and spore counts were performed (Fig. 3a and b). Sessile cell counts varied from 4.8 ± 0.3 log CFU/cm² for the attachment stage (bacterial adhesion) and up to 10.2 ± 0.3 log CFU/cm² at the biofilm maturation stage (Table 1). The average sessile bacterial cell count per day is shown in Table 1 and Fig. 3a. The highest values were observed on the 5th day (biofilm maturation) with counts of 10.2 ± 0.3 log CFU/cm² for BHI-B12 (Exp. 1), 9.7 ± 0.2 log CFU/cm² for BHI-B12-W (Exp. 2), 8.7 ± 0.4 log CFU/cm² for BHI-B12-C (Exp. 3), and 9.0 ± 0.2 log CFU/cm² for BHI-B12-W/C (Exp. 4).

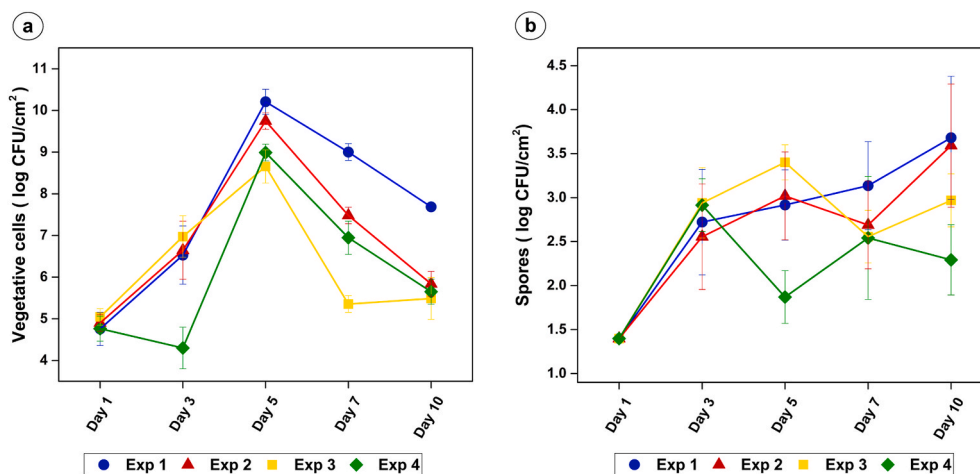


Fig. 3. Counting of sessile cells and spores in the *B. sporothermodurans* biofilm life cycle: sessile cells (a) and spores (b) on SS surface. Exp. 1: BHI-B12 (Control Group); Exp. 2: BHI-B12-W; Exp. 3: BHI-B12-C; and Exp. 4: BHI-B12-W/C.

Table 1

Mean values of comparison of the biofilm life cycle.

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Day 1	4.8 ± 0.4	4.9 ± 0.2	5.0 ± 0.2	4.8 ± 0.3
Day 3	6.5 ± 0.7 ^a	6.6 ± 0.7 ^{a b}	6.9 ± 0.5 ^b	4.3 ± 0.5 ^c
Day 5	10.2 ± 0.3	9.7 ± 0.2	8.7 ± 0.4	9.0 ± 0.2
Day 7	9.0 ± 0.2 ^a	7.5 ± 0.2 ^{b d}	5.3 ± 0.2 ^{c d}	6.9 ± 0.4 ^d
Day 10	7.7 ± 0.1 ^a	5.8 ± 0.3 ^b	5.5 ± 0.5 ^b	5.6 ± 0.3 ^b

*Values (log CFU/cm²) and standard deviation followed by same letters in the lines do not differ in the Tukey test ($p > 0.05$). Exp. 1: BHI-B12 (Control Group); Exp. 2: BHI-B12-Whey; Exp. 3: BHI-B12-Casein; and Exp. 4: BHI-B12-Whey/Casein.

cm² for BHI-B12-W/C (Exp. 4). Passive biofilm dispersal was observed from the 7th and 10th days for all substrates. The results indicated no significant differences ($p > 0.05$) for average sessile cell count among the experiments for Day 1 (Attachment biofilm) and Day 5 (Maturation biofilm). However, significant differences were found on days 3, 7, and 10 ($p < 0.05$). Sporulation was observed on the 3rd day. The spore counts varied from <1.4 (1st day) to 3.7 log ± 0.3 log spores/cm² (10th day) (Fig. 3b). Regarding the spore count, no significant differences were observed for all experiments ($p > 0.05$) (Fig. 3b).

All these results indicate that *B. sporothermodurans* can grow in different dairy substrates and that milk proteins (whey, casein) affect biofilm formation on stainless steel, specifically, in Experiments 2, 3 and 4, when compared to the control group. Low *B. sporothermodurans* cell counts in the adhesion stage were observed in Exp. 4 (Day 3). However, this scenario did not prevent the biofilm from reaching maturity (Day 5), which suggests that proteins have influenced bacterial behavior. This finding is in agreement with previous studies showing that proteins reduce the adhesion of other bacteria, such as *L. monocytogenes*, *S. aureus*, *S. marcescens*, *Pseudomonas fragi* and *Escherichia coli* (Barnes et al., 1999; Yun et al., 2014).

B. sporothermodurans mono-species biofilm, with counts of 3.10 ± 0.02 log CFU/cm², and a multispecies biofilm of *Geobacillus stearothermophilus* and *B. licheniformis* with 5.1 ± 0.02 log CFU/cm² on SS surfaces have previously been reported in the literature. The initial inoculum was 10⁷ CFU/mL and counting occurred after 3 days (Jindal et al., 2016). The values for the mono-species biofilm are lower than those reported in this work (minimum: 4.3 ± 0.7, Exp. 4 and maximum 7.0 ± 0.7, Exp. 3) for the same time. However, a different disaggregation method, which included swabs and a vortex (Jindal et al., 2016), was used. The large number of biofilm cells reported in this work could likely be associated with entrapment and fouling. In addition, the methodology of this work (ultrasonic bath and microscopy) has shown a higher efficiency in disaggregating and harvesting sessile cells (Hamilton et al., 2009).

Other studies have reported biofilm formation of other spore-forming HRS bacilli and thermophilic bacteria (Jindal & Anand, 2018; Kilic & Coleri Cihan, 2020; Parkar et al., 2001). Species such as *Bacillus pumilus* can form a biofilm with 5.9 log CFU/cm² on SS surfaces after 2 days, reaching similar values to those reported in this study (Kilic & Coleri Cihan, 2020). Spores of *Bacillus*, including thermophilic bacilli, can attach more easily onto SS surfaces when compared to vegetative cells (Parkar et al., 2001). The same behavior was also observed for *B. sporothermodurans*, *B. licheniformis* and *G. stearothermophilus* spores (Jindal & Anand, 2018). Furthermore, corrosion is inevitable after prolonged exposure to these species (Gupta & Anand, 2018). Therefore, the presence of spores in the dairy industry is a concern, since they are resistant to severe processing conditions, and display thermal and sanitizer resistance, while also contributing to equipment corrosion and pitting (Gupta & Anand, 2018; Li et al., 2020; Wan et al., 2018).

3.3. Characterization of *B. sporothermodurans* biofilm formation by scanning electron microscopy (SEM)

Despite having significant differences ($p > 0.05$) in the cell counts and among biofilms in all four experiments, it is possible that milk proteins contribute to changes in biofilm phenotype. To explain the behavior of *B. sporothermodurans* and the influence of milk proteins (whey and casein), we performed SEM measurements of the biofilm-covered area of the samples on the 1st day (cell attachment), 5th day (biofilm maturation) and 10th day (biofilm dispersal).

1st day – The measured area shows significant differences for the first day ($p < 0.05$). In particular, biofilm areas in Exp. 3 and 4 were significantly different compared to the others, but not between them (Fig. 4c, d, 4e). Thus, SEM revealed that this behavior may have been influenced by casein, present in both experiments. In Exp. 2 (Fig. 4b) the shapes of the observed surface-adhered biofilms exhibited an unusual distribution, with small clusters and circular patterns growing in-plane horizontal direction. The mean, median, minimum and maximum values of the biofilm areas are consistent with this finding (Supplementary Table A.1).

Interestingly, significant differences ($p < 0.05$) in cell diameters were also observed, especially for Exp. 4 (Supplementary Fig. A.1a); the minimum, average, and maximum values were 0.6 µm, 0.8 ± 0.02 µm, and 0.9 µm, respectively. On the other hand, Exp. 1, 2 and 3 exhibited minimum values of 0.4 µm, an average of 0.6 ± 0.01 µm, and a maximum of 0.9 µm (Supplementary Fig. A.1a). Regarding *B. sporothermodurans* length on biofilm surfaces, values ranged from a minimum of 2.5 µm to a maximum of 26.9 µm (Supplementary Fig. A.1b). The average lengths for Exp. 1, 3 and 4 were 7.0 ± 0.4 µm, 7.0 ± 0.7 µm, 7.5 ± 0.5 µm, respectively, and with no significant differences on the 1st day ($p > 0.05$). However, the average length for Exp. 2 was 10.9 ± 0.4 µm ($p < 0.05$).

So far, only planktonic cells of *B. sporothermodurans* have been examined with SEM, with lengths in the range of 6 µm–10 µm and diameters of 0.5 µm (Montanari et al., 2004). Those values are lower in comparison to the maximum values observed for sessile cells reported in this work (lengths up to ~ 27 µm and widths ~ 0.9 µm). Nonetheless, a possible justification for the diameter increase in this microorganism may be related to the presence of central or subterminal spores. Planktonic cells with subterminal or central spores can present a cylindrical shape, from 0.7 µm to 0.8 µm wide (Montanari et al., 2004). Moreover, we can speculate that the increase of cell diameter and covered area can be due to higher interaction between cells and the contact surface, which might result in cell deformation.

The length values found in this work (up to ~ 27 µm) show that this microorganism may be able to have elongated or filamentous cells. These elongated or filamentous cells are a central characteristic of biofilm formation, preceding the motility and fragmentary biofilms (Anbumani et al., 2021; Janissen et al., 2015; Liu et al., 2020). *Xylella fastidiosa* growth was elongated by up to 10 times their typical size when connected to neighboring clusters in the biofilm formation (Janissen et al., 2015). Other studies with *B. cereus*, *Vibrio cholerae*, *Caulobacter crescentus*, and *P. aeruginosa* have also reported these filamentous cells (Heinrich et al., 2019; Liu et al., 2020; Wucher et al., 2019; Yoon et al., 2011). On the other hand, bacterial cell dimensions, cell attachment and biofilm structure can also be affected by the chemical composition of the culture medium (Berne et al., 2018; Gloag et al., 2020). The differences in cell attachment observed in the spatial distribution of Exp.3 and 4 can be explained by the cell interactions with these chemical compounds. These interactions may be a concern for food safety, since these proteins are frequently in contact with surfaces in dairy production (Flint et al., 2015).

5th day – Remarkably, mature biofilms exhibited significant differences when compared to cell attachment results (1st day - Supplementary Fig. A.1a) and among experiments on the 5th day. The milk proteins (whey protein, casein) affected the final number of sessile cells present

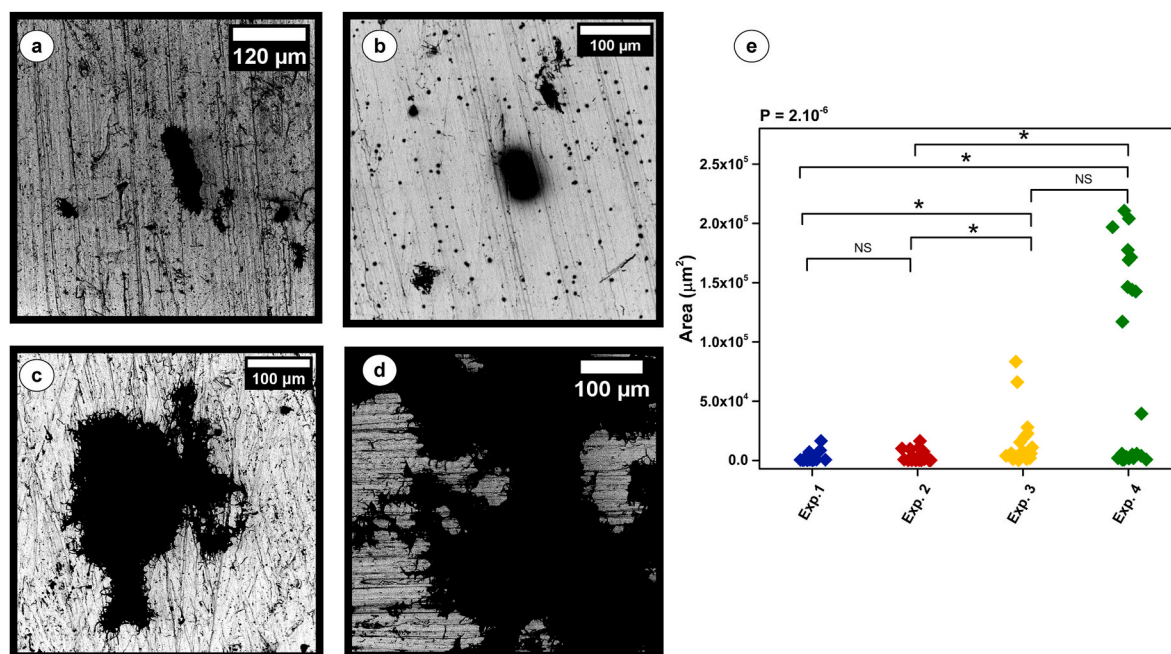


Fig. 4. Characterization by SEM of *B. sporothermodurans* biofilm on SS surface on the 1st day. (a) Exp. 1: BHI-B12 (Control Group); (b) Exp. 2: BHI-B12-W; (c) Exp. 3: BHI-B12-C; and (d) Exp. 4: BHI-B12-W/C; (e) Comparison of clusters or aggregates on SS surface; * indicate that differences between values are significantly different ($p < 0.05$); and n.s. indicate no significant differences.

on SS surfaces (Results shown in section 3.2), and SEM data also showed a heterogeneous behavior (Fig. 5b, c and 5d), indicating that milk proteins possibly impact on biofilm formation.

The size of the niches in biofilm maturation were significantly higher in Exp. 1 - control group (Fig. 5a), showing significant differences ($p < 0.05$) compared to Exp. 2 (Fig. 5b, e and 5f), Exp. 3 (Fig. 5c, e and 5f), and Exp. 4 (Fig. 5d and e). In general, *B. sporothermodurans* behavior in the control group followed the expected biofilm maturation (5th day),

with higher niches than in cell attachment (1st day). This result concurs with our previous findings, which showed an increase in the number of vegetative cells (Section 3.2). The comparative analysis is also evident in the mean, median, maximum and minimum values of the biofilm areas (Supplementary Table A.2). We were able to visualize and measure individual cells under high magnification for all groups, except for Exp. 2. The averages were $6.2 \pm 0.8 \mu\text{m}$, $6.4 \pm 0.4 \mu\text{m}$, and $3.6 \pm 0.2 \mu\text{m}$ for length, and $0.5 \pm 0.03 \mu\text{m}$, $0.7 \pm 0.02 \mu\text{m}$, and $0.8 \pm 0.05 \mu\text{m}$ for

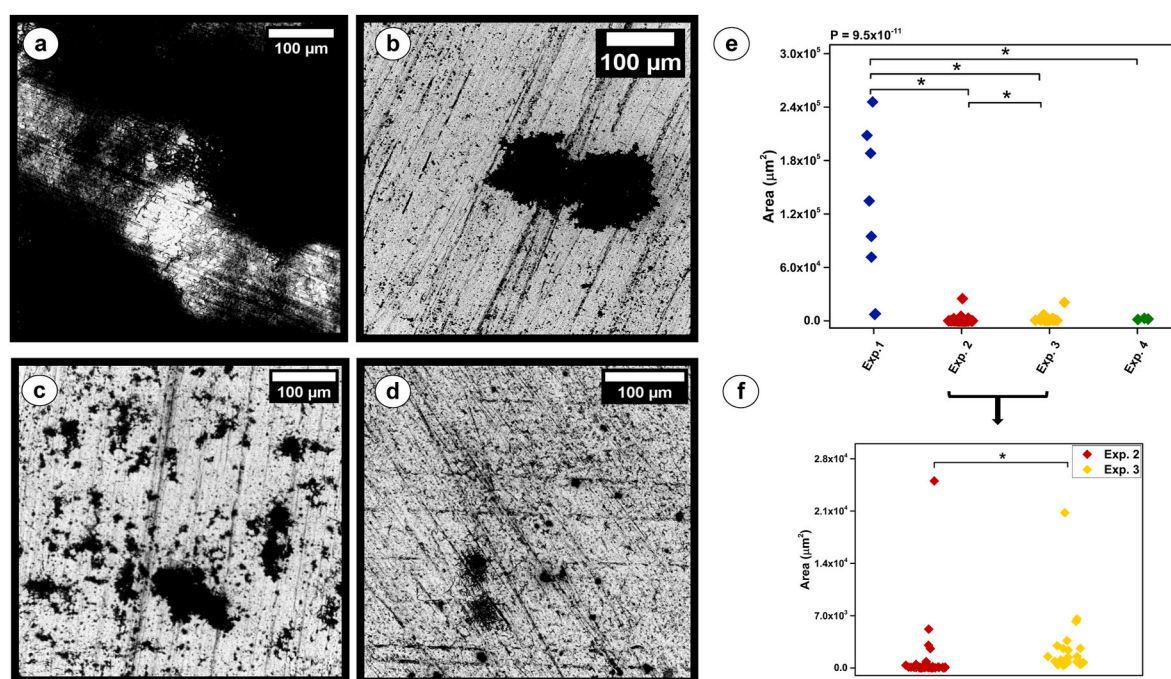


Fig. 5. Characterization by SEM of *B. sporothermodurans* biofilm on SS surface on the 5th day. (a) Exp. 1: BHI-B12 (Control Group); (b) Exp. 2: BHI-B12-W; (c) Exp. 3: BHI-B12-C; (d) Exp. 4: BHI-B12-W/C; (e) Comparison of clusters or aggregates on SS surface; (f) Comparison of clusters between the Exp. 2 and Exp.3; * indicate that differences between values are significantly different ($p < 0.05$).

diameter, in Exp. 1, Exp. 3, and Exp. 4, respectively. Moreover, circular patterns of agglomerates in Exp. 2 (Supplementary Fig. A.2a, A.2b) confirmed our results for the 1st day, Fig. 4b. Indeed, we were able to see *B. sporothermodurans* underneath these structures (Supplementary Fig. A.2b).

Our results indicate that milk proteins affect *B. sporothermodurans* structure on SS surfaces, and consequently, the matrix of extracellular polymeric substances (EPS) that contribute to the biofilm life cycle (Karygianni et al., 2020). EPS composition depends on the microorganism, local shear stress, nutrient availability, and host environment (Karygianni et al., 2020). Although EPS is common in most bacteria, different structural formats and distributions are exhibited by Gram-positive bacteria, including secondary wall polymers covalently linked to peptidoglycan, glycoconjugate, glycosylated proteins, capsular polysaccharides, and secreted exopolysaccharides (Whitfield et al., 2020).

The biofilm spatial distribution (Fig. 5b, c, d and Supplementary Fig. A.2a, b) on the 5th day could be considered as dispersion. The counts of sessile cells (results presented in section 3.2) show that the biofilm reached its maximum growth on the 5th day for all experiments. When comparing these data with the microscopic observations, a large number of small biofilm niches is revealed in the active dispersal stage. In this case, the bacteria spread out on stainless steel, with a few and small clusters. This process is triggered by signals (quorum sensing, starvation or cell death) and is characterized by an orderly transition (McDougald et al., 2012). This dispersal, also known as native, is initiated by the bacteria that are located at the center of mature biofilms (Rumbaugh & Sauer, 2020). Therefore, active dispersion is only noticed by microscopy. These results indicate that the use of more than one technique to quantify adhered cells and to observe biofilm architecture is extremely useful (Azeredo et al., 2017), and encourages further publications on the minimum information guidelines of biofilms (Allkja et al., 2020; Coenye et al., 2020; Lourenço et al., 2014).

10th day - A reduction of surface-adhered biofilm sizes was observed in Exp. 1 - Control group. Visually, most of the observed clusters showed detachment and erosion (Fig. 6a and Supplementary Fig. A.3a, b, c, d, e). These observations can also be confirmed by niche measurements

(Supplementary Table A.3). The stage at which the biofilm spatial distribution culminates in cell detachment is known as passive dispersal, and usually occurs at the end of life cycles and is characterized by cell detachment and erosion (McDougald et al., 2012). Unlike the active dispersion that was observed on the 5th day by SEM only, this passive dispersion could be confirmed by the plate count, a behavior expected for the 10th day. The spatial distribution of Exp. 1 (Fig. 6a) was significantly different ($p < 0.05$; Fig. 6e) when compared to Exp. 2 (Fig. 6b) and Exp. 3 (Fig. 6c). In Exp. 2 we notice an increase of agglomerates when compared to other days, although small clusters remained on the SS surface (Fig. 6b, and Supplementary Fig. A.3f, g, h). In addition, differences ($p < 0.05$) in cell length and diameter were also observed on the 10th day (Supplementary Fig. A.4a, b). Other peculiarities were observed on the 10th day (Supplementary Fig. A.3f, g, h, i, j), including small niche formats (Supplementary Fig. A.3f, g, h, i, j). However, more detailed studies to chemically characterize these small niches have yet to be carried out. In Exp. 3, bacteria had long connections (Supplementary Fig. A.3k, l) and structures that form part of the biofilm spatial distribution (Supplementary Fig. A.3m, n, o). These elongated cells were also observed in Experiment 4 (Supplementary Fig. A.3p, q), along with cells involved in EPS (Supplementary Fig. A.3r - green circles). The structures had a circular shape, which was the same shape observed in Exp. 1 (Supplementary Fig. A.3b, c, d, e - blue arrow) and Exp.2 (Supplementary Fig. A.3i - red arrow).

A schematic diagram summarizing the main results of the *B. sporothermodurans* biofilm life cycle is shown in Fig. 7 and Supplementary Fig. A.5.

Although we have seen elongated cells in this study, we cannot conclude what they represent. In the literature, the elongated cells are a common behavior of planktonic cells in HRS strains, previously observed by conventional optical microscopes (Pettersson et al., 1996). However, for *X. fastidiosa* this behavior is more common in sessile cells located at the edges of the biofilms. This is because the elongated bacteria are responsible for interconnecting small bacterial clusters in the biofilm (Janissen et al., 2015). Chains of elongated cells with minimal visible septation are found on the edges of the expanding bacterial population. This has already been reported for *Campylobacter jejuni* in

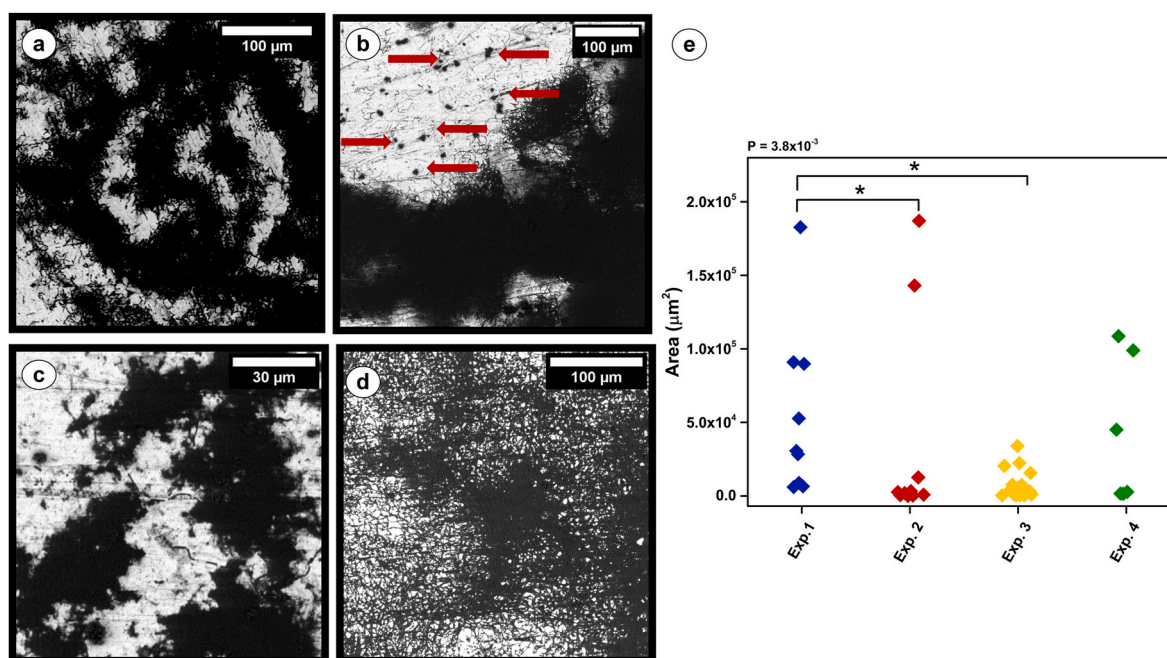


Fig. 6. Characterization by SEM of *B. Sporothermodurans* biofilm on SS surface on the 10th day. (a) Exp. 1: BHI-B12 (Control Group); (b) Exp. 2: BHI-B12-W; (c) Exp. 3: BHI-B12-C; (d) Exp. 4: BHI-B12-W/C; (e) Comparison of clusters or aggregates on SS surface; * indicate that differences between values are significantly different ($p < 0.05$).

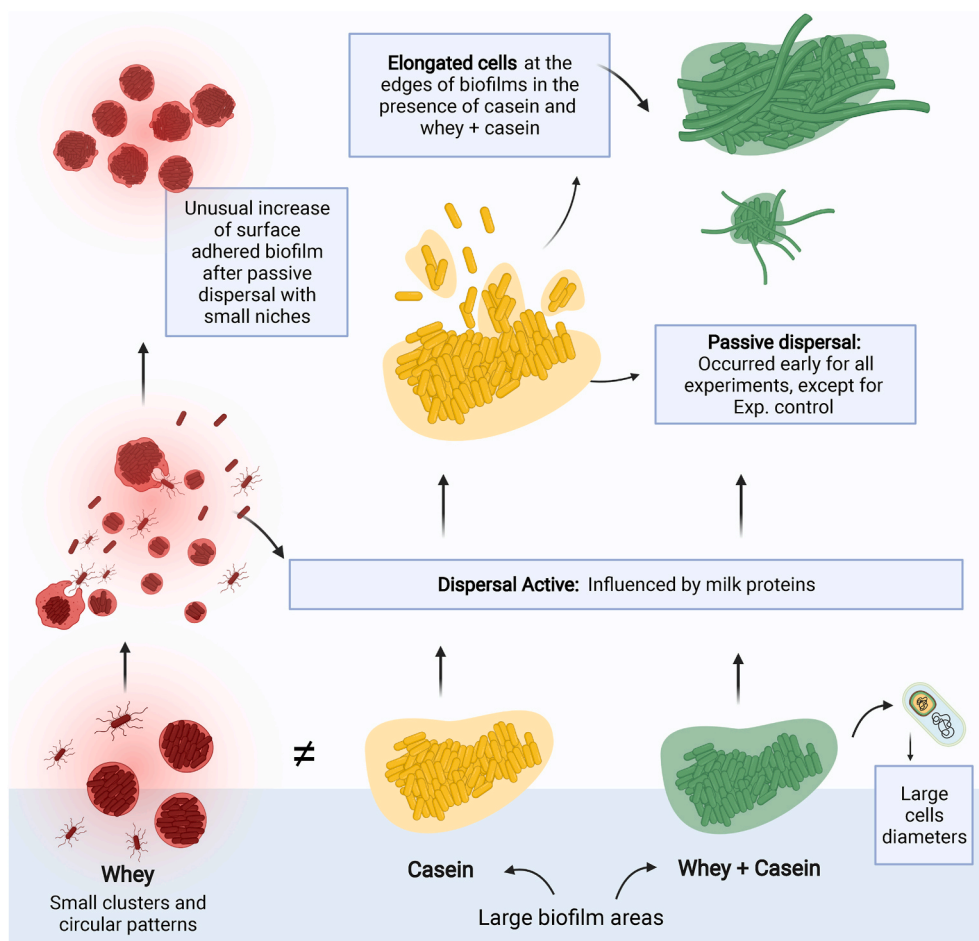


Fig. 7. Schematic diagram summarizing the main results of *B. sporothermodurans* biofilm life cycle.

the presence of milk and blood (Liu et al., 2020), and for *X. fastidiosa* biofilms (Janissen et al., 2015). Moreover, overproduction of proteins (GpSB and YpsA) may favor filamentation, or the formation of elongated cells through the disruption of FtsZ assembly (a ring-like structure and central division protein) (Amos & Lowe, 1998; Brzozowski et al., 2020; White & Eswara, 2020). This elongation is interesting for research groups that study non-model organisms, as they may reveal new regulatory mechanisms (White & Eswara, 2020).

The homogeneity of SS samples was verified before biofilm formation, and none of the samples displayed cracks or trenches. However, biofilm layers were observed to be overlaying surface cracks or trenches in Exp. 4 (Supplementary Fig. A.3s, t). The corrosion and pitting of SS surfaces might shelter undesirable bacteria (spoilage and pathogenic), putting the production line at risk. The SS surface pitting caused by these bacteria has already been reported previously (Gupta & Anand, 2018). Microbially-induced corrosion (MIC) is a field of study with high interest for the dairy industry (Gupta & Anand, 2018; Li et al., 2019, 2020).

In addition to the conditioning film influence on surface-adhered biofilms, biofilm formation at the air-liquid surface also takes place (Supplementary Fig. A.6a, b, c, d), which might be related to pairwise competition between biofilms, either for oxygen or nutrients (Koza et al., 2020). This formation may be related to the adaptive lineages, allowing for interaction with the environment and showing an improved fitness advantage (Koza et al., 2020). Moreover, physiological and morphological adaptations, common at the air-liquid interface for biofilms, can trigger metabolic changes and sporulation. These cells are metabolically more active and aggressive in interactions and coordinated actions against competitors (Caro-Astorga et al., 2020). One possibility is that competition between biofilms influences the counts of

sessile cells (Fig. 3a). Therefore, for *in vitro* studies, we recommend that future works take a broader approach to accompany this formation, including both microscopy and counting. Assays for biofilm formation at the air-liquid surface is also interesting for studies regarding the production and comparison of EPS (Hölscher et al., 2015; Martin et al., 2020). Biofilms at the air-liquid interface tend to be thicker and are frequent in industrial storage and piping systems, mainly due to being partly filled during operation or residual liquid remaining after a production cycle (Ostrov et al., 2019; Wijman et al., 2007).

Other bacilli, such as *B. cereus*, *B. subtilis* and *B. licheniformis* associated with milk also form biofilms at the air-liquid interface with a robust pellicle (Martin et al., 2017; Ostrov et al., 2019; Yan et al., 2017). Moreover, *B. cereus* presents better biofilm formation at the air-liquid interface than in submerged systems (Wijman et al., 2007). We did not find any studies that report the formation of *B. sporothermodurans* in this system. Thus, we suggest that future studies evaluate this micro-organism further, as it may be present in storage and industrial pipelines, or remaining in residual liquid after a production cycle (Wijman et al., 2007).

3.4. Evaluation of enzymatic treatments and disinfectants against *B. sporothermodurans* biofilms by single tube method (STM)

The Single Tube Method (ASTM E2871) was the standard method selected to measure the effectiveness of antimicrobial products against biofilms, which is determined by a log reduction of viable cell counts. The initial inoculum was increased from 10^4 to 10^8 CFU/mL to verify if the reduction of viable cells would reach the expected values (~ 5 log). Despite the inoculum being under different conditions from the previous

experiments, a well-developed biofilm coverage can be seen in [Supplementary Fig. A.7](#). The evaluation was performed after 24 h of biofilm formation in BHI–B12 medium. The log value of the untreated control (LD_C) and treated surfaces (LD_T), as well as the LR of enzymatic products and sanitizers used, are shown in [Table 2](#) and [Supplementary Fig. A.7](#). The XPS analysis is shown in [Supplementary Fig. A.8](#).

Biojet + Enzyjet Plus are enzymatic products recommended for open surfaces, whereas Enzycip is applied on Clean-in-place Systems (CIP) ([iTram Higiene, 2020b](#)). The enzymatic products (iTram®) were effective for *B. sporothermodurans* biofilm removal on SS surfaces ([Table 2](#) and [Supplementary Fig. A.7a, b, c, d, e](#)). It is important to note that enzymatic products are not bactericidal; they only breakdown proteinaceous materials and polysaccharide matrices ([Anand et al., 2014](#)). Thus, iTram® application protocols recommend that the enzymatic products must be applied prior to a disinfectant ([iTram Higiene, 2020b](#)). As the methodology of this work evaluated only the effectiveness of products and not the cleaning process, it is expected that when applied together with the other steps, a higher level of removal would be achieved than the presented data ([iTram Higiene, 2020b](#)). In addition, the synergistic effect of enzymes (amylase, protease and lipase) at a neutral pH against biofilm formation in the food industry has already been demonstrated in a previous work ([Tsiaprazi-Stamou et al., 2019](#)), as well as in a dairy-filling hose ([Fysun et al., 2019](#)).

The ECOLAB® chemicals were efficient in eliminating biofilms on the SS surface ([Table 2](#) and [Supplementary Fig. A.7f, g, h, i](#)). The chemical composition of both products is a blend of different concentrations of acetic and peracetic acid, octanoic, and hydrogen peroxides. These products are recommended to food and beverage industries for the disinfection of stainless steel surfaces (pipes, tanks, fillers, evaporators, and pasteurizers) ([ECOLAB, 2020b; 2020a](#)).

The chemical composition of the very first layers found on the surface were identified using XPS analysis, both before (Control) and after the STM procedure. The survey spectra ([Supplementary Fig. A.8](#)) for all samples after treatment showed no significant trace of remaining organic matter on the surface. All spectra indicated a chemical composition identical to the Control sample, which characterizes the effectiveness of the disinfection cleaning procedure. Therefore, these products can be used as a strategy to prevent and reduce the impact of *B. sporothermodurans* biofilms. The enzymatic treatment and disinfection efficacy on *B. sporothermodurans* biofilms was the last step of this research. In future developments, a detailed investigation will be required to evaluate the sporicidal activity, which must use reliable methodologies such as Quantitative Carrier Test (QCT - Standard ASTM E2111), Sporicidal Activity Test (SAT - AOAC 966.04), or the Three Step Method (TSM - ASTM E2414) ([Parker et al., 2018](#)).

4. Conclusion

This is the first report of the *B. sporothermodurans* biofilm life cycle under the influence of milk proteins (whey and caseins). In this study, the steps of cell attachment, maturation and dispersal until sporulation were observed through sessile cell and spore counting. Moreover, measurements of bacterial cells, biofilm size and spatial distribution were performed using SEM.

B. sporothermodurans on SS surfaces showed a large number of biofilms. The bacterial count was influenced by milk proteins, which also modified spatial distributions and cell dimensions/morphology. Biofilm-related characteristics, such as elongated and interlaced cells, eventual pitting or corrosion, and biofilm formation at the air-liquid interface, were also observed.

An interesting strategy to prevent and reduce the harmful impact of these biofilms in the dairy industry could be the use of enzymatic products and disinfectants, such as those evaluated in this work. In addition, we suggest that future research also evaluate the sporulation of these microorganisms.

New strategies, combining experimental and theoretical analysis, as

Table 2

Effectiveness of enzymatic treatment and disinfectants on *B. sporothermodurans* biofilm.

	Enzymatic treatment (iTram®)				Disinfectants (Ecolab®)			
	Biojet + Enzyjet Plus		Enzycip		Vortex		Vortex ES	
	LD	SD	LD	SD	LD	SD	LD	SD
Treated sample (LD _T)	3.83	0.39	3.06	0.57	3.62	0.37	2.42	0.36
Control sample (LD _C)	8.86	0.18	8.14	0.10	8.13	0.13	8.74	0.03
Log10 reduction (LR)	5.03	0.43	5.08	0.57	4.51	0.39	6.32	0.36

LD: log10 density; SD: Standard deviation.

well as innovative techniques can benefit future studies. Microscopy of three-dimensional space and time by automated analysis, which quantifies high-yield images may contribute to the evaluation of phenotypic and genotypic variations of biofilms. In addition, these tools and protocols help to predict biofilm inhibitory activity and quantify microbial communities by providing a solid quantitative basis.

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CRediT authorship contribution statement

Vanessa Pereira Perez Alonso: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Rodrigo Cezar de Campos Ferreira:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Mônica Alonso Cotta:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition. **Dirce Yorika Kabuki:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that this manuscript has not been submitted to, nor is under review at, another journal or other publishing venue. None authors have affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2021.108743>.

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