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מניעת התפתחות עמידויות בקטריאליות ושיפור תהליכי ניקוי של משטחי העבודה בתעשיית החלב

תקציר (בעברית)

מיקרואורגניזמים הנצמדים לצנרת החליבה חייבים להיות מוסרים בתהליך הניקוי, דבר הדורש שימוש בחומרי ניקוי ובאנרגיה לסחרור החומר. בעבודה זו נבחנה יעילות הניקוי של חומרים עם תוספות של תרכובות כלור כלפי תבדידים בקטריאליים אשר בודדו מסביבת ייצור החלב. תוצאות המחקר מצביעות כי חומרים אשר מכילים תרכובות על בסיס כלור ומצטיינים בחיטוי הם אינם בהכרח טובים לתהליכי הניקוי במיוחד כלפי תבדידי החלב. בהמשך, מצאנו כי יכולת חיידקים ליצור ביופילם משפיעה על יעילות תהליך הניקוי. נראה כי תנאים האופייניים לסביבה הקשורה לייצור החלב עשויים להגביר סלקציה לזני חיידקים בעלי יכולות הישרדות מוגברות לעיבוד התעשייתי ולתהליכי הניקוי בתעשיית החלב. זנים אלו עברו אפיון פיזיולוגי ומורפולוגי במסגרת מחקר זה והתוצאות מצביעות על כך שלזנים ישנה יכולת מוגברת ליצור ביופילם בהשוואה לחיידקים מעבדתיים. בנוסף, נמצא כי הזנים מפגינים עמידות מוגברת בפני תהליכי ניקוי אשר אמורים להבטיח את ניקיון סביבת ייצור החלב.

תקציר (באנגלית)

Surface attached microorganisms are often involved in contamination of dairy products and therefore present a major microbiological challenge in the field of food quality and safety. One of the main strategies for maintaining the optimal hygiene level in dairy processing facilities is regular cleaning and disinfection. However, it appears that the dairy-associated *Bacillus* strains are characterized by formation of robust biofilms during growth in milk. Furthermore, the dairy-associated *Bacillus* isolates demonstrate higher resistance to cleaning procedures compared to non-dairy *Bacillus*. We therefore suggest that the enhanced resistance of the dairy *Bacillus* isolates can be attributed to robust biofilm formation. Findings of the study underline the importance of evaluating the efficiency of commercial cleaning agents towards biofilm-forming resistant bacteria, which are relevant to industrial conditions. Consequently, we believe that these findings will facilitate assessing and improving industrial cleaning procedures.

מבוא ותיאור הבעיה

נזקים מיקרוביאליים מהווים איום עיקרי לאיכות ובטיחות של חלב ומוצריו. נזקים אלו כוללים בדרך כלל פירוק של חלבונים ושומנים על ידי אנזימים שמיקרואורגניזמים מפרישים בחלב. מיקרואורגניזמים המתבססים על משטחי עבודה (צנרת, מיכלים ואביזרי חליבה) ברפתות ובמחלבות מהווים בעיה חמורה בתחום איכות ובטיחות של חלב ומוצריו. משטחי העבודה בתעשיית החלב בנויים ממספר חומרים הבאים במגע עם העטין ועם החלב. החומרים העיקריים כוללים: פלדת אל חלד (פלב"מ), גומי, סיליקון וחומרים פלסטיים מורכבים. חיידקים המצויים בחלב גולמי נצמדים לשטח פני המשטחים במערכת החליבה. עיקר ההיצמדות מתחולל כאשר המערכת נמצאת במצב סטטי. במערכת החליבה החלב

נמצא בדרך כלל בתהליך זרימה, עם הפסקות בין דבוקת פרות אחת לשנייה. חיידקים יכולים להיצמד בזמן הזרימה ובהפסקות אל דפנות הצנרת. לאחר היצמדות ראשונית למשטח, מתחיל תהליך התיישבות החיידקים ובניית ביופילם. חיידקי הביופילם (תאים וגטטיביים ונבגים) במתקני החליבה מהווים מקור לזיהום מיקרוביאלי מתמשך שמהווה מצד אחד איום לבטיחות של חלב ומוצריו ומצד שני, גורם להקטנת אורך חיי המדף של המוצרים ע"י חיידקי קלקול (spoilage). תהליך הפסטור משמיד את מרבית החיידקים הוגטטיביים בחלב, אולם אינו פוגע בנבגים. בנוסף לפגיעה אפשרית באיכות החלב, הזיהום המיקרוביאלי עלול לגרום לנזק כלכלי משמעותי עקב פגיעה בציוד החליבה. חיידקי הביופילם מקטלזים ריאקציות כימיות וביולוגיות הגורמות לקורוזיה מתכתית של הצנרת, המיכלים ואביזרי החליבה. ביופילמים הנוצרים על משטחים הבאים במגע עם החלב כוללים, בין השאר, חיידקי בצילוס (*Bacillus*). חיידקים אלו מסוגלים להתרבות גם בטמפרטורות המקרר, לייצר אנזימים המפרקים את מרכיבי החלב ואף לייצר טוקסינים. תופעות אלו מאיצות את קלקול מוצרי החלב וגורמות לנזק כלכלי.

חיידקי הבצילוס הם חיידקים גרם חיוביים יוצרי נבגים המהווים את המיקרופלורה הדומיננטית בביופילמים שנוצרים במערכות המשמשות את משק החלב. הביופילמים של בצילוס יכולים להכיל הן תאים וגטטיביים והן נבגים. כמות הנבגים עולה כאשר הביופילם חשוף לאוויר, מצב המתרחש בצנרת החלב במהלך הפסקות החליבה. פרסומים דיווחו שביופילם של בצילוס החשוף לאוויר מורכב בעיקר מנבגים, כלומר המטריקס הפולימרי של הביופילם משמש כמוקד להבשלת נבגים אשר עלולים להשתחרר ממנו ולגרום לזיהום מתמשך של סביבת הייצור. מכיוון שהנבגים עמידים הרבה יותר מתאים ווגטטיביים לטיפול בחומרי חיטוי הם יישארו במערכת החליבה, ישרדו את הטיפול התרמי (פסטור) ויזהמו את החלב.

חיידקים שנצמדים לצנרת החליבה חייבים להיות מוסרים בתהליך הניקוי, דבר הדורש שימוש בחומרי ניקוי ובאנרגיה לסחרור החומר. ניקוי המערכת חייב להיות יעיל, חסכוני ככל האפשר במים, דטרגנטים ואנרגיה (חום, שאיבה, ואקום וכו'). פעולות ניקוי נכונות ויעילות, המשאירות אביזרי החליבה נקיים לאחר מהלך הניקוי, משפיעות משמעותית על איכות החלב המופק במכון החליבה. מרכיבי מכון החליבה נחשפים במהלך הניקוי לזרם של חומרי ניקוי. תפקידם של חומרים אלו לסלק את חיידקי הביופילם יחד עם שאריות החלב הדבוקות למשטחים ולמנוע הידבקות חוזרת. לפיכך, יש לוודא את יעילות הניקוי של החומרים ופעילותם לסילוק הביופילם. בעבודה מחקר נוכחית, מטרתנו הייתה ללמוד על ההשפעה והתרומה של תרכובות מבוססי כלור על תהליכי ניקוי שאר נועדו למנוע זיהומים במערכות החליבה.

<u>מסקנות המחקר</u>

יכולת היווצרות ביופילמים עשויה להקנות לחיידקים עמידות מוגברת בפני טיפולים אנטימיקרוביאליים וכך להשפיע על יעילות תהליך הניקוי.

תנאי סביבה הקשורים לייצור החלב עשויים להגביר סלקציה לזני חיידקים בעלי יכולות הישרדות מוגברות לעיבוד התעשייתי ולתהליכי הניקוי בתעשיית החלב.

לזנים עמידים עלולה להיות יכולת מוגברת ליצור ביופילם בהשוואה לחיידקים מעבדתיים והבסיס לכך הינו בשינויים גנטיים המובילים ביכולת אדפטציה גבוהה לתנאי סביבה שבזמן ייצור החלב.

מצ"ב שני מאמרים מדעיים שנבעו כתוצאה מהמחקר. יודגש כי בפרק התודות של המאמרים צוין מקור המימון.

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Robust Biofilm-Forming *Bacillus* Isolates from the Dairy Environment Demonstrate an Enhanced Resistance to Cleaning-in-Place Procedures

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Abstract: One of the main strategies for maintaining the optimal hygiene level in dairy processing facilities is regular cleaning and disinfection, which is incorporated in the cleaning-in-place (CIP) regimes. However, a frail point of the CIP procedures is their variable efficiency in eliminating biofilm bacteria. In the present study, we evaluated the susceptibility of strong biofilm-forming dairy *Bacillus* isolates to industrial cleaning procedures using two differently designed model systems. According to our results, the dairy-associated *Bacillus* isolates demonstrate a higher resistance to CIP procedures, compared to the non-dairy strain of *B. subtilis*. Notably, the tested dairy isolates are highly persistent to different parameters of the CIP operations, including the turbulent flow of liquid (up to 1 log), as well as the cleaning and disinfecting effects of commercial detergents (up to 2.3 log). Moreover, our observations indicate an enhanced resistance of poly-γ-glutamic acid (PGA)-overproducing *B. subtilis*, which produces high amounts of proteinaceous extracellular matrix, to the CIP procedures (about 0.7 log, compared to the wild-type non-dairy strain of *B. subtilis*). We therefore suggest that the enhanced resistance to the CIP procedures by the dairy *Bacillus* isolates can be attributed to robust biofilm formation. In addition, this study underlines the importance of evaluating the efficiency of commercial cleaning agents in relation to strong biofilm-forming bacteria, which are relevant to industrial conditions. Consequently, we believe that the findings of this study can facilitate the assessment and refining of the industrial CIP procedures.

Keywords: dairy industry; biofilm; *Bacillus* species; biofilm derived spores; cleaning-in-place; disinfecting effect

1. Introduction

Microbial contamination, caused by biofilm-forming bacteria, is one of the main threats to the quality, safety, stability and nutritional value of dairy products [1,2]. Moreover, biofilms are not only a potential source of contamination; they can also increase the corrosion rate of equipment used in the milk industry, impair heat transfer, and increase fluid frictional resistance [3]. Therefore, controlling biofilm formation is of major importance to the dairy industry [4–6].

Members of the *Bacillus* genus are among the most commonly found biofilm-formers in dairy farms and processing plants [7–9]. In addition to aggressive biofilm, these bacteria are able to form heat-resistant endospores [10,11]. To this end, the biofilm matrix can serve as an epicenter for the ripening of spores, which can be released from it and cause continuous contamination of the production environment [12,13]. Spores, as well as biofilm cells, are highly resistant to antimicrobial agents, which makes it rather difficult to eliminate them [11,14]. Moreover, biofilm matrix offers additional

protection for embedded endospores, allowing their survival and colonization in the surrounding environment, when conditions are favorable [15]. In *B. subtilis*, the matrix has two main components, an exopolysaccharide (EPS) and amyloid-like fibers. Another extracellular polymer, γ-poly-DL-glutamic acid (PGA), is produced in copious amounts by some *B. subtilis* strains [16–18].

The main strategy to prevent biofilm formation, applied in the dairy industry, is to clean and disinfect regularly before bacteria attach firmly to surfaces [19,20]. Cleaning and disinfection in dairy processing plants have been incorporated into the cleaning-in-place (CIP) regimes, which include regular cleaning of processing equipment with alkaline and acidic liquids at high temperatures and flow velocities [4,21,22]. However, a weak point of CIP processes, evident in both industrial- and laboratory-scale systems, is their variable efficiency in eliminating established biofilms [4,21,23]. It is conceivable that biofilm formation can facilitate bacterial adaptation and survival in certain environmental niches. We therefore hypothesized that aggressive biofilm formation by dairy-associated bacteria might increase their resistance to industrial cleaning procedures.

In the present study, we evaluated the susceptibility of strong biofilm-forming dairy *Bacillus* isolates to cleaning-in-place procedures using two different model systems, which resemble industrial cleaning conditions. Our results show that the dairy-associated *Bacillus* isolates demonstrate enhanced resistance to different aspects of the CIP procedures, including mechanical, chemo-biological and disinfecting effects. Such reduced susceptibility can be attributed to robust biofilm formation by the tested dairy *Bacillus*.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The following bacterial strains were used in this study: (i) dairy-associated isolates, such as *B. paralicheniformis* S127 [24,25], *B. licheniformis* MS310, *B. subtilis* MS302, *B. paralicheniformis* MS303 [24]; (ii) non-dairy isolate *B. subtilis* NCIB3610 (descendant of *B. subtilis* Marburg); (iii) poly-γ-glutamic acid (PGA)-overproducing mutant derivatives of *B. subtilis* 3610, *B. subtilis* YC295 (Δ*ywcC*) and *B. subtilis* YY54 (Δ*pgdS*) (a gift of Y. Chai [18]). *B. licheniformis* MS310, *B. subtilis* MS302 and *B. paralicheniformis* MS303 whole-genome shotgun projects are deposited at DDBJ/EMBL/GenBank, under accession numbers MIPQ00000000, MIZD00000000, MIZE00000000 respectively.

For routine growth, the strains were propagated in Lysogeny broth (LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter, pH 7) or on a solidified LB medium, supplemented with 1.5% agar at 37 °C.

2.2. Generation of Biofilm-Derived Spores

Biofilm colonies were generated at 30 °C in a biofilm-promoting medium (LBGM = LB + 1% *v*/*v* glycerol + 0.1 mM $MnSO_4$) [26]. Biofilm-derived spores were obtained from colonies, as described previously [21]. Briefly, the grown (three-day-old) colonies, harvested and suspended in phosphate buffered saline (PBS; 0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl per 200 mL, Sigma Aldrich, St. Louis, MO, USA), were disrupted by mild sonication (Vibra Cell, Sonics, Newtown, CT, USA; amplitude 60%, pulse 10 s, pause 10 s, duration 2 min, instrument power: 7.2 Joules per second). During sonication, the samples were kept on ice. Then, heat killing was performed at 80 °C for 20 min. Cell numbers after heat killing were quantified by the spread plating method.

2.3. Staining Extracellular Matrix of Biofilm-Derived Spores

Biofilm-derived spores were stained using the FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain (Molecular Probes, Eugene, OR, USA), according to the manufacturer's protocol. Stained samples were visualized by confocal laser scanning microscopy (CLSM; Olympus IX81, Tokyo, Japan) at a 10 μm scale.

2.4. Preparation for Cleaning Tests and Enumeration of Biofilm-Derived Spores

The preparation of biofilm-derived spores for cleaning tests was performed, as described in the previous study [21]. Briefly, 200-µL aliquots of the spore suspension (containing approximately two million spores) were applied in the sampling area of stainless-steel sampling plates and dried in a biological laminar hood for 1 h. Two sampling plates were not exposed to the cleaning procedures (control). Following each cleaning test, the sampling plates were immediately subjected to abundant rinsing with tap water at RT (similar to the CIP procedures at Israeli dairy farms, where the rinsing with water stage is introduced after applying a cleaning agent). For the enumeration of the spores, the sampling area on each plate was carefully swabbed with cotton swabs, moistened in PBS buffer. Swabs from each plate were then agitated in PBS in separate test tubes. Serial dilutions from each sample were prepared, followed by spread plating on LB agar for CFU analysis. Plates were incubated for 24 h at 37 °C, before the colonies were counted. The efficiency of a cleaning procedure was evaluated by comparing the number of viable spores (attached to sampling plates), before and after cleaning.

2.5. Cleaning Solutions

The following cleaning solutions were used in this study: Caustic soda (NaOH), sodium hypochlorite (NaOCl) and six different commercial alkaline detergents, defined as solutions I (10–15% NaOH, 3–5% NaOCl), A (polycarboxylate, phosphates, 3.6% NaOCl), M (>5% polycarboxylate, 5–15% phosphates, 3.6% NaOCl), F (5% phosphonates, polycarboxylates), D (active chlorine, alkaline-based) and H (active chlorine, phosphates, additives, alkaline-based), which are commonly used in the Israeli dairy farms. The pH value of the tested solutions varied between 11–12; the pH of NaOH was 13; and the pH of NaOCl was 4. In accordance with the manufacturer's recommendations, the agents were used at the following concentrations: (i) 0.5% (*v*/*v*) for solutions A, M, F, D, H; (ii) 0.6% (*v*/*v*) for solution I; (iii) 0.5% (*m*/*v*) for caustic soda and detergent H; (iv) 0.018% (*v*/*v*) for sodium hypochlorite (similar to the NaOCl concentration in working solutions of the examined cleaning agents, such as A, M and I). As a control, tap water was used (pH value around 7.7), with a standard level of hardness (50 mg/L Ca^{2+}, 50 mg/L Mg^{2+}), without the addition of any detergent.

2.6. Cleaning Test Installations

The cleaning tests were carried out either using the cleaning-in-place (CIP) model system (closely resembling the typical conditions for milking systems) [21] or using the simplified laboratory procedure, developed in this study.

2.6.1. CIP Model System

The main components of the CIP model system were described in the previous study [21]. In brief, the system consists of a 5-m stainless-steel milk line (fitted with a test unit) for pumping the cleaning agents from the basin, milk releaser, and a stainless-steel return line to the basin. The test unit has T-junctions, protruding 35, 125 or 275 mm from the main loop, reflecting different degrees of cleaning difficulty. Sampling plates with the spores were mounted on the T-junctions and cleaned in the installation. The temperature of the cleaning solution during the cleaning tests was 50 °C. To generate flushing pulsation of the circulating liquid, air was introduced into the system every 8 s. The duration of each cleaning cycle was 10 min.

2.6.2. Laboratory System

For cleaning tests in the laboratory system, sampling plates with the spores were placed into 100 mL plastic vessels (Yoel Naim, Rehovot, Israel), containing 50 mL of cleaning solution (preliminarily warmed to 50 °C). The samples were incubated in closed vessels at conditions simulating those in the CIP-model system (50 °C, 250 rpm) for 10 min.

2.7. Evaluation of the Effect of the Cleaning Agents on the Viability of Bacillus Spores

The tested solutions were added to spore suspension within tap water containing around 1×10^7 CFU/mL spores. The spore suspension without the addition of detergents was used as a control. The samples were incubated in closed tubes under the conditions of the laboratory system (50 °C, 250 rpm) for 10 min. The CFU measurements of the number of viable spores were made immediately after the addition of the tested cleaning agents and following 10 min of incubation.

2.8. Statistical Analysis

The results of the study are the means and standard deviation (SD) of at least two independent biological experiments, performed in triplicate. The Student's *t* test was used to calculate the significance of the difference between the mean expression of a given experimental sample and the control sample. A p value of <0.05 was considered significant.

3. Results

3.1. Dairy-Associated Bacillus Isolates Exhibit Robust Biofilm Phenotype Compared to B. subtilis 3610

We focused this investigation on biofilm-forming milk isolates of *Bacillus* species, which were obtained from Israeli dairy farms and recently identified and characterized [24]. The isolates were further characterized using a colony-type biofilm model for the robustness of their biofilm-forming capabilities (Figure 1; Table S1). We found notable differences in the colony-biofilm phenotype between *B. subtilis* 3610 and the dairy *Bacillus* isolates (Figure 1A). Thus, the biofilm colonies of *B. subtilis* 3610 had a complex "wrinkled" structure (shown to be a network of channels rich in biofilm matrix-producing cells [27,28]), but were not mucoid. The colonies of the tested dairy-associated strains combined an intricate "wrinkled" phenotype with the formation of highly mucoid "channel"- and "ridge"-like structures, not observed for *B. subtilis* 3610 (Figure 1A).

To support this observation, we analyzed the extracellular matrix content in the colony biofilm of the tested dairy *Bacillus* isolates and *B. subtilis* 3610 by visualizing matrix proteins. Our results indicate that biofilm cells/spores, harvested from colonies of the dairy-associated strains, could be surrounded by higher amounts of extracellular polymeric substances (EPS), compared to *B. subtilis* 3610 (Figure 1B).

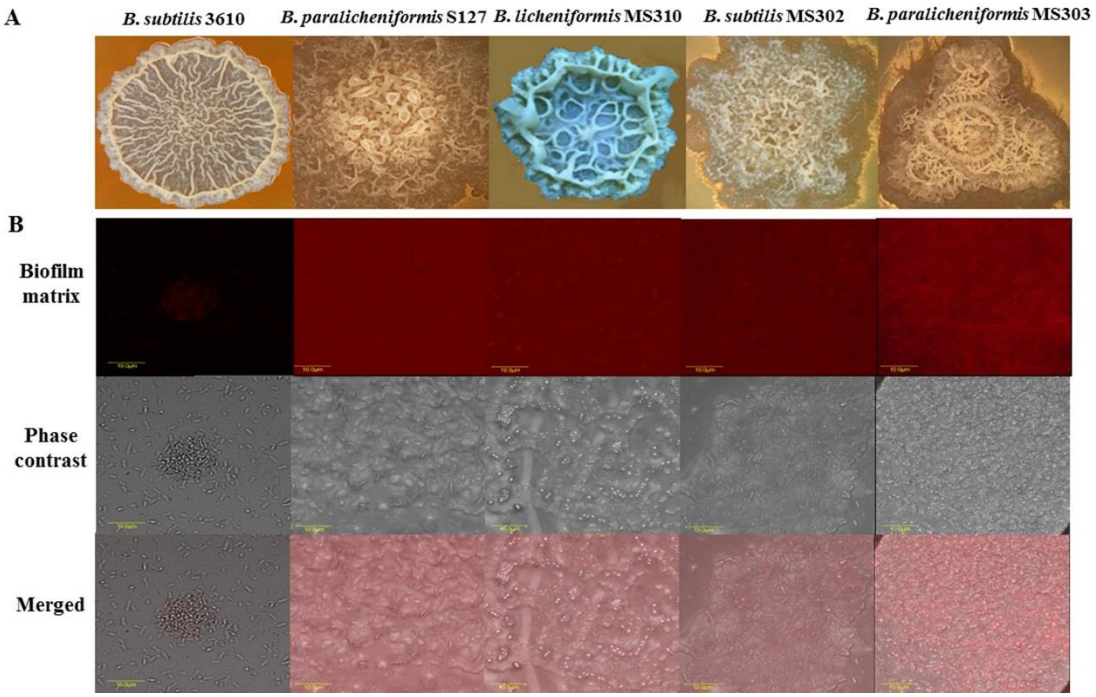

Figure 1. Dairy-associated *Bacillus* isolates exhibit robust biofilm formation. (**A**) Colony type biofilm formation by the tested *Bacillus* strains in the biofilm-promoting medium, LBGM. The images were taken using a stereoscopic microscope (Zeiss Stemi 2000-C; Carl Zeiss, Gottingen, Germany). (**B**) Biofilm-derived spores of the dairy *Bacillus* strains are surrounded by high amounts of the extracellular matrix. Protein components of the biofilm matrix were stained red. The samples were analyzed using a confocal laser scanning microscope (CSLM, Olympus, Japan). Scale: 10 µm.

3.2. Dairy-Associated Bacillus Isolates Display an Enhanced Resistance to the Mechanical Effect of Water Circulation

Primarily, we evaluated the susceptibility of the tested strains to water circulation in the CIP model system (closely resembling the conditions typical for milking pipes). Cleaning with water alone reflects the mechanical cleaning effect brought about by the flow of liquid in the installation [21,29]. The susceptibility of the dairy-associated *Bacillus* strains to cleaning procedures was compared to the non-dairy isolate *B. subtilis* 3610 (used as a model strain in our previous study [20]). In order to simulate dairy biofilm, we used a system that is based on the biofilm-derived spores of the tested *Bacillus*, obtained from the biofilm colonies as previously described [21].

We found that the biofilm-derived spores of the dairy *Bacillus* were significantly (by 0.3–1 log) more resistant to water circulation, compared to *B. subtilis* 3610, in the case of 35 and 125 mm T-junctions (representing high levels of turbulence; Figure 2). In the samples placed into the 275-mm T-junctions (the lowest degree of turbulence available in the CIP model system), the susceptibility to cleaning was either similar (*B. paralicheniformis* S127) or lower by 0.1–0.3 log (*B. paralicheniformis* MS303, *B. licheniformis* MS310, *B. subtilis* MS302) than the control samples.

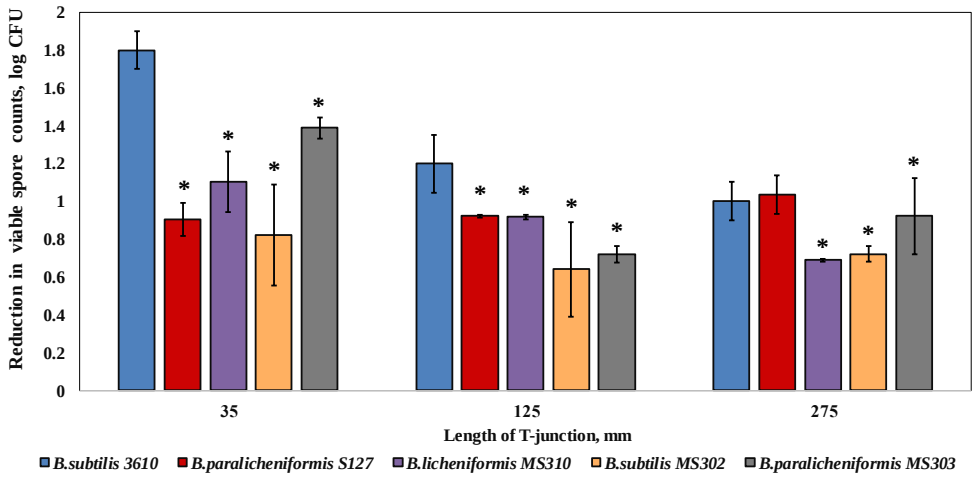

Figure 2. Effect of the cleaning procedure with tap water on the removal of biofilm-derived spores of the dairy-associated *Bacillus* in the CIP model system. Sampling plates, each containing approximately 2 million spores of *B. subtilis* 3610 or dairy *Bacillus* isolates, were mounted on T-junctions, protruding 35, 125, and 275 mm from the main loop of the CIP model system, and cleaned in the installation. Tap water, without the addition of any detergent, was used as the cleaning agent. A basic assumption was the similar adhesion efficiency of the spores of each tested strain in different experimental repeats (since the spores were obtained using previously validated experimental procedures [21]). The cleaning effect was evaluated by comparing the number of viable spores (attached to the sampling plates), before and after cleaning. The results represent the means and standard deviations (SD) of two independent biological experiments, performed in triplicate. * Statistically significant difference ($p < 0.05$) between the reduction in the viable spore counts of a given sample and the reduction in the spore counts for *B. subtilis* 3610 (control).

Next, we wanted to test the persistence of the examined *Bacillus* strains against the chemical effect of the commercial cleaning solutions. Since the chemical effect of the cleaning agents is less dependent on the flow turbulence, it was decided to simplify our experimental system to a lab-scale cleaning test (hereinafter referred to as the laboratory system). We first confirmed the validity of this system by comparing the strains' ability to withstand a mechanical effect. Importantly, the dairy-associated *Bacillus* demonstrated an enhanced resistance to water circulation (by 0.6–0.7 log), compared to *B. subtilis* 3610, also during the cleaning tests performed in the laboratory system (Figure S1). A strong correlation between the results obtained in the two differently designed experimental systems indicates the reliability of the approach used.

3.3. Dairy-Associated Bacillus Isolates Demonstrate an Enhanced Resistance to Commercial Cleaning Agents during CIP Procedures

Next, we evaluated the susceptibility to commercial cleaning agents of two selected dairy-associated isolates, *B. paralicheniformis* S127 and *B. licheniformis* MS310, which demonstrated the highest amount of EPS surrounding biofilm bacteria, according to a relative fluorescence analysis, in comparison to *B. subtilis* 3610 (Table S1). Consequently, we performed cleaning procedures using six different alkaline detergents, caustic soda (NaOH) and sodium hypochlorite (NaOCl) at concentrations recommended by the manufacturers. It was found that *B. licheniformis* MS310, as well as *B. paralicheniformis* S127, were more resistant to the tested solutions (up to 2.3 and 0.76 log, respectively), compared to *B. subtilis* 3610 (Figure 3). Interestingly, *B. subtilis* 3610 was particularly susceptible to agents I, M, D and H, whereas *B. paralicheniformis* S127 was highly persistent to cleaning with agent H and NaOH, but similarly susceptible to solutions I, M and F as *B. subtilis* 3610. *B. lichenifomis* MS310 was exceedingly resistant to treatment by the examined solutions, especially to agents I, M and H (Figure 3).

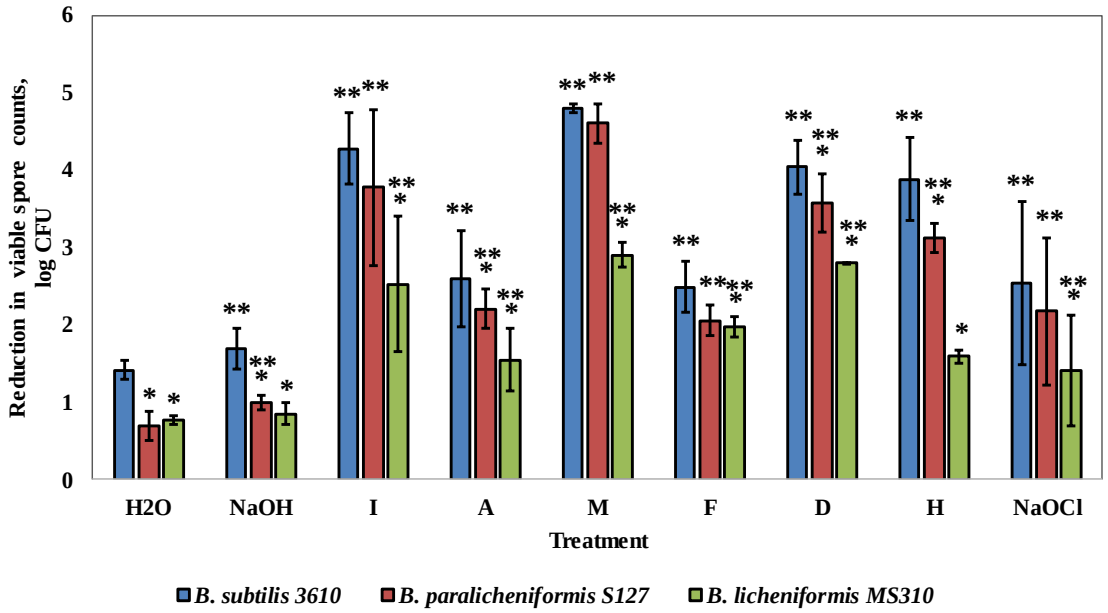

Figure 3. Effect of commercial cleaning agents on the removal of the biofilm-derived spores of the dairy-associated *Bacillus* in the simplified laboratory system. Sampling plates, each maintaining approximately 2 million spores of the tested *Bacillus* strains, were cleaned in the laboratory system. Caustic soda, sodium hypochlorite and the following cleaning solutions—I, A, M, F, D and H (compositions and dosages are described in Methods)—were used as the cleaning agents. The cleaning effect was evaluated by comparing the numbers of viable spores (attached to sampling plates), before and after cleaning. The results represent the means and standard deviation (SD) of two independent biological experiments, performed in triplicate. * Statistically significant difference ($p < 0.05$) between the reduction in the viable spore counts in a given sample and the reduction in the spore counts for *B. subtilis* 3610 (control). ** Statistically significant difference ($p < 0.05$) between the reduction in the viable spore counts, after treatment with a given cleaning agent, and the reduction in the spore counts for the same strain, after incubation with tap water.

As indicated in the previous study [21], the biofilm removal effect of a cleaning agent includes both the mechanical effect of the liquid circulation and the chemo-biological effect from the active components, present in the agent. To gain greater insight into the mode of action of the examined solutions, we calculated their chemo-biological effect in relation to the biofilm-derived spores of the tested strains. As shown in Figure S2, *B. lichenifomis* MS310 was significantly more resistant to the chemo-biological effect of the examined solutions, compared to the other strains. At the same time, in most cases, *B. paralichenifomis* S127 was equally susceptible to the chemo-biological effect, compared to 3610. This indicates that the tested strains have varying degrees of resistance to the mechanical and chemo-biological effects of cleaning agents. Thus, the low susceptibility of MS310 to the examined solutions results from the increased resistance both to their mechanical and chemo-biological effect (Figure S3). In the case of S127, a high resistance to the majority of the tested solutions (NaOH, I, F, D) is caused mainly by the low susceptibility to the mechanical removal of spores, while the persistence to agents A and H results from a reduced sensitivity to both the mechanical and chemo-biological impacts (Figure 3; Figure S3).

3.4. Dairy-Associated Bacillus Isolates Demonstrate an Enhanced Resistance to the Disinfecting Effect of the Tested Agents

Primarily, we determined the ability of the tested agents to remove surface-attached spores, without affecting the viability (cleaning effect) and/or inactivating the spores (disinfecting effect). For this, spore suspensions were incubated with each of the tested agents under the conditions of the laboratory system. We found that the examined agents had different influences on the viability of the

biofilm-derived spores of the tested strains (Figure 4). Thus, solutions D and M notably reduced the spore counts of *B. subtilis* 3610, after 10 min of incubation (Figure 4); there was a 0.5 log reduction in the viable spores for S127, after incubation with solution I; while none of the tested solutions affected the viability of the MS310 spores. Interestingly, NaOCl, commonly used as a disinfecting agent, did not influence the viability of the tested strains at the examined concentration (the dosage widely used in industrial cleaning agents; Figure 4).

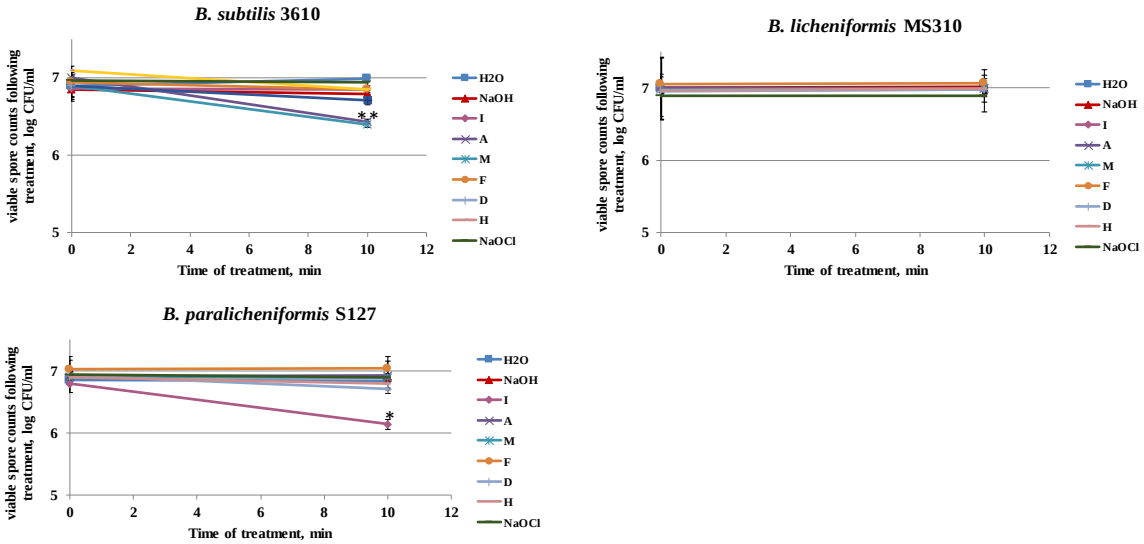

Figure 4. Effect of the examined cleaning agents on the viability of the biofilm-derived spores of the tested *Bacillus* strains. Caustic soda, sodium hypochlorite, and different cleaning solutions—I, A, M, F, D, and H (compositions are described in Methods)—were added to the tubes, with spore suspension of the tested *Bacillus* isolates. Spore suspension, without any detergent, was used as the control. The effect on spore viability was evaluated by comparing the numbers of viable spores in the control and after the treatment with the tested agents (following 10 min of incubation at 50 °C, 250 rpm). The results represent the means and standard deviation (SD) of two independent biological experiments, performed in duplicate. * Statistically significant difference ($p < 0.05$) between the viable spore counts in a given sample versus the spore counts after cleaning with water (control).

Next, we determined a correlation between the cleaning and disinfecting effects of the tested detergents. Thus, we defined the ability of a cleaning agent to reduce the number of viable spores after 10 min of a cleaning cycle, as a disinfecting effect. We compared the percentage of the disinfecting effect to the total chemo-biological effect of a cleaning agent (taken as 100%). The difference between the total chemo-biological effect of the tested agent and the disinfecting effect was defined as the cleaning effect [21]. As can be inferred from Figure 5, the ratio between the cleaning and disinfecting effects of the examined detergents differed for the tested strains. Thus, the removal of the MS310 spores was due solely to the cleaning effect of the tested solutions. *B. paralicheniformis* S127 was significantly more resistant to the disinfecting effect of agents A, M, F, H, and NaOH, compared to *B. subtilis* 3610, but much more susceptible to the disinfecting effect of solution I (Figure 5). Overall, the chemo-biological effect of the tested agents was mostly due to the removal of surface-attached spores (cleaning effect) and not to disinfecting.

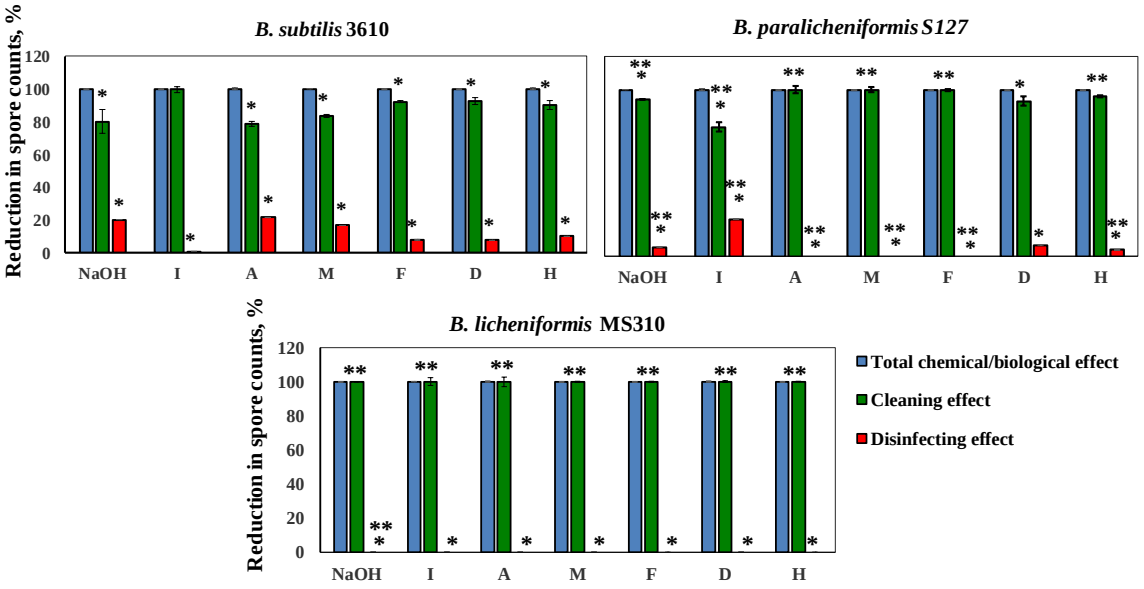

Figure 5. Correlation between the cleaning and disinfecting effects of the examined agents for each tested strain. Caustic soda and different cleaning solutions—I, A, M, F, D, and H (compositions are described in Methods)—were added to the tubes, with spore suspension, of the tested *Bacillus* isolates and incubated for 10 min at 50 °C, 250 rpm. The ability of a cleaning agent to reduce the number of viable spores was defined as the disinfecting effect. The percentage of the disinfecting effect was compared to the total chemical/biological effect of a cleaning agent (taken as 100%). The difference between the total chemical/biological effect of a cleaning agent and the disinfecting effect was defined as the cleaning effect. The results represent the means and standard deviation (SD) of two independent biological experiments, performed in duplicate. * Statistically significant difference ($p < 0.05$) between the reduction in the spore counts due to the cleaning or disinfecting effects versus the total chemo-biological effect of a tested agent. ** Statistically significant difference ($p < 0.05$) between the reduction in the viable spore counts in a given sample and the reduction in the spore counts for *B. subtilis* 3610 (control).

4. Discussion

It becomes increasingly clear that biofilm formation by *Bacillus* species can facilitate their survival in the dairy environment [11,21]. Our current study investigated the effect of CIP procedures on strong biofilm-forming dairy *Bacillus*, compared to the non-dairy *B. subtilis* 3610, using differently designed model systems. As in our previous study [21], we used biofilm-derived spores to simulate the type of hygiene problem common in practice. Thus, similarly to actual dairy biofilm, biofilm-derived spores combine the presence of biofilm matrix [21] and a high content of spores [29,30]. Moreover, the resistance of vegetative cells/spores to cleaning and disinfection can be greatly enhanced by the presence of EPS [21,31]. At the same time, the presence of spores within the *Bacillus* biofilm may also modify biofilm properties, e.g., interaction forces [12].

In the current study, two model systems were used to ensure that the enhanced resistance of the dairy isolates to cleaning procedures is observed under different experimental conditions, which are relevant to the industrial CIP systems. Moreover, the design of the CIP system, employed in our previous study does not allow for the evaluation of the disinfecting effect of the cleaning agents on *Bacillus* spores directly in this system [21]. The laboratory system, developed in this study, provides sufficient conditions both for determining the mechanical, chemo-biological and disinfecting effects of the cleaning agents.

A first notable finding of the study was the enhanced resistance of the dairy *Bacillus* to the mechanical effect of liquid circulation. Thus, the most expressed difference in cleaning susceptibility between the dairy-associated strains and *B. subtilis* 3610 was observed at high levels of turbulence (35- and 125-mm T-junctions, CIP model system; Figure 2). In the case of a lower turbulence (275-mm

T-junction), the difference between the dairy *Bacillus* isolates and the non-dairy strain is markedly decreased, and for some strains, it was insignificant (Figure 2). These results suggest that the protective effect of *Bacillus* biofilm matrix is most strongly expressed under a high turbulence of liquid flow. Previous studies demonstrate that a high turbulence may facilitate the removal of surface-attached bacteria [21,32–34], but may also increase the rate of attachment by bringing the microbial cells and the substrate in close proximity [35]. Thus, biofilm formation by the dairy-associated *Bacillus* can be detrimental not only in so-called "dead legs" (equipment details, in which the flow of liquid is significantly less turbulent), but also in main pipelines.

Furthermore, we showed that the biofilm-derived spores of the dairy *Bacillus* isolates are much more resistant to commercial cleaning agents, compared to *B. subtilis* 3610. Presumably, the causes of this resistance differ between the tested strains. Thus, the biofilm-derived spores of MS310 are, apparently, less susceptible both to the mechanical and chemo-biological effects of the employed solutions (Figures S2 and S3). At the same time, *B. paralicheniformis* S127 has the highest resistance to the mechanical removal of spores but shows a variable susceptibility to the chemo-biological effect of the tested agents.

As shown in our previous study [21], the chemo-biological effect of cleaning agents comprises a disinfecting effect (inactivating bacteria) and/or removal of them from the surfaces of dairy equipment (cleaning effect). According to our results, the dairy *Bacillus* isolates are significantly less susceptible to the disinfecting effect of the tested agents, compared to the non-dairy strain (except solution I in the case of S127; Figure 4; Figure 5). The observed differences in the mechanical and chemo-biological effects between the tested strains might be explained by the dissimilarities in the biofilm structure. For instance, a correlation between colony biofilm phenotype of the tested strains, and their resistance to the cleaning procedures, was observed (Figure 1). Thus, the dairy-associated *Bacillus*, characterized by a mucoid biofilm phenotype, were less susceptible to mechanical and chemo-biological effects during the CIP procedures. Since biofilm matrix components can be responsible for binding and/or neutralizing detergents and antimicrobial agents [36,37], differences in the matrix structure/composition can lead to differences in cleaning and/or disinfection susceptibility. Thereby, the biofilm matrix composition was shown to affect the susceptibility of food-associated staphylococci to cleaning and disinfection agents, with polysaccharide matrix-producing strains being more resistant to the lethal effect of benzalkonium chloride [38]. Likewise, the efficiency of monochloramine disinfection was dependent on the quantity and composition of EPS in *Pseudomonas* biofilms. Protein-based EPS-producing *P. putida* was less sensitive to monochloramine than polysaccharide-based EPS-producing *P. aeruginosa*, since monochloramine had a selective reactivity with proteins over polysaccharides [39]. According to Bridier et al. (2011) [40], the biofilm of the *P. aeruginosa* clinical isolate, in which a high delay of benzalkonium chloride penetration is recorded, was characterized by a large quantity of proteinacious matrix. Moreover, the authors report that, in *P. aeruginosa*, resistance to antimicrobial agents is intimately related to the inherent three-dimensional organization of cells into the exopolymeric matrix. Therefore, the low sensitivity of the dairy *Bacillus* isolates to the CIP procedures (compared to *B. subtilis* 3610) may be connected to differences in the structure/composition of the biofilm matrix.

Importantly, mucoid colony formation, observed for the dairy *Bacillus* isolates, was viewed as a hallmark of poly-γ-glutamic acid (PGA) production in multiple previous studies [17,18]. Significant production of PGA could result in a stronger attachment to surfaces due to its adhesive properties [41]. To this end, PGA-overproducing derivatives of *B. subtilis* 3610 (*B. subtilis* YC295 and *B. subtilis* YY54) were significantly more resistant to the mechanical effect of water circulation, compared to the wild type (Figure 6C). Notably, biofilm colonies of these mutant strains were more mucoid, compared to the WT (Figure 6A). Moreover, the biofilm-derived spores of PGA-overproducing *B. subtilis* were surrounded by higher amounts of proteinaceous extracellular matrix, which resembles the tested dairy *Bacillus* isolates (Figure 6B). Therefore, the presence of PGA in the biofilm matrix of the examined bacterial strains may be one of the factors enhancing resistance to the CIP procedures.

We believe that the role of PGA and other presumptive EPS components of the dairy-associated *Bacillus* in relation to cleaning and disinfecting agents is an important subject for further investigation.

Relatively low cleaning and, especially, disinfecting effects of the tested solutions (Figure 5) might lead to undesirable implications regarding the hygiene level in dairy environments. For instance, the rapid recovery of biofilms after inappropriate disinfectant treatment is often observed. This may be due to the re-growth of surviving cells, residual biofilm, providing a conditioning layer for further cell attachment, or the selection of resistant microorganisms that survive and thrive after antimicrobial treatment [5]. In addition, biofilm cells exposure to low (sub-lethal) concentrations of disinfecting compounds, including chlorine-based detergents, can stimulate further biofilm development [10,42,43]. Therefore, we speculate that the composition of commercial CIP agents should be revised and evaluated under the experimental conditions suggested in this study.

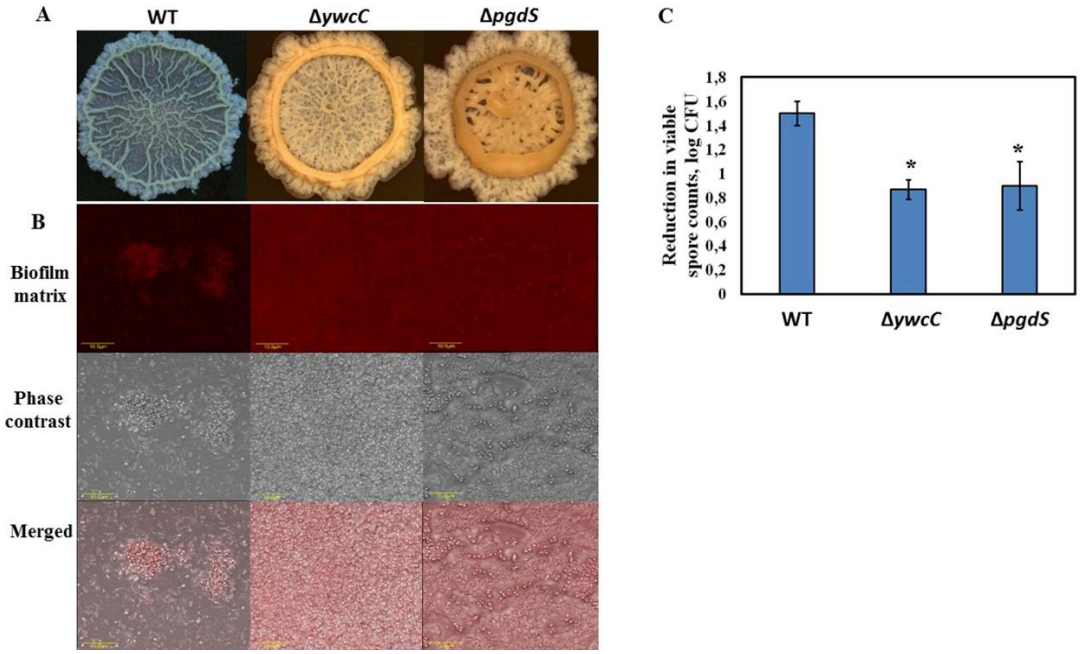

Figure 6. PGA-overproducing derivatives of *B. subtilis* 3610 exhibit increased resistance to the CIP procedures due to enhanced biofilm formation. (**A**) Colony biofilm formation by the tested *Bacillus* strains in the biofilm-promoting medium, LBGM. The images were taken using a stereoscopic microscope (Zeiss Stemi 2000-C; Carl Zeiss, Gottingen, Germany). (**B**) Biofilm-derived spores of the PGA-overproducing *B. subtilis* strains are surrounded by high amounts of extracellular matrix. Protein components of the biofilm matrix were stained red. The samples were analyzed using a confocal laser scanning microscope (CSLM, Olympus, Japan). Scale: 10 μm. (**C**) The effect of water circulation on the removal of biofilm-derived spores of the PGA-overproducing derivatives of *B. subtilis* 3610 in the laboratory CIP system. * Statistically significant difference ($p < 0.05$) between the reduction in the viable spore counts in a given sample and the reduction in the spore counts for *B. subtilis* 3610 (control).

5. Conclusions

We demonstrated in this study that the dairy-associated *Bacillus* isolates are characterized by an enhanced resistance to different aspects of the CIP procedures, such as the mechanical, chemo-biological, and disinfecting effects, compared to the non-dairy *Bacillus*. Such increased resistance can be attributed to robust biofilm formation by the tested dairy *Bacillus*. The results of the study underline the importance of revising the composition of commercial cleaning agents and evaluating their efficiency in relation to strong biofilm-forming bacteria, relevant to industrial conditions. To this end, the biofilm-derived spores of the dairy-associated *Bacillus*, examined in this study, can be used as an appropriate model for assessing and refining the CIP procedures.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/8/4/134/s1, Figure S1: Effect of the cleaning procedure with tap water on removal of the biofilm-derived spores of the dairy-associated *Bacillus* in the simplified laboratory system, Figure S2: Chemo-biological effect of the commercial cleaning agents on removal of the biofilm derived spores in the laboratory CIP system, Figure S3: Correlation between mechanical and chemo-biological effect of the examined agents in relation to the removal of the biofilm derived spores in the laboratory CIP system, Table S1: Relative quantity of the matrix, surrounding biofilm-derived spores of the dairy-associated *Bacillus* isolates and *B. subtilis* 3610.

Author Contributions: Conceptualization, I.O. and M.S.; methodology, I.O.; investigation, I.O. and T.P.; data curation, I.O.; writing—original draft preparation, I.O.; writing—review & editing, M.S.; supervision, M.S.; funding acquisition, M.S.

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Conflicts of Interest: The authors declare no conflict of interest.

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Adaptation of *Bacillus* species to dairy associated environment facilitates their biofilm forming ability

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ABSTRACT

Biofilm-forming *Bacillus* species are often involved in contamination of dairy products and therefore present a major microbiological challenge in the field of food quality and safety. In this study, we sequenced and analyzed the genomes of milk- and non-milk-derived *Bacillus* strains, and evaluated their biofilm-formation potential in milk. Unlike non-dairy *Bacillus* isolates, the dairy-associated *Bacillus* strains were characterized by formation of robust submerged and air–liquid interface biofilm (pellicle) during growth in milk. Moreover, genome comparison analysis revealed notable differences in putative biofilm-associated determinants between the dairy and non-dairy *Bacillus* isolates, which correlated with biofilm phenotype. These results suggest that biofilm formation by *Bacillus* species might represent a presumable adaptation strategy to the dairy environment.

1. Introduction

Members of *Bacillus* genus are among the most commonly found bacteria in dairy farms and processing plants (Sharma and Anand, 2002; Simoes et al., 2010). *Bacillus* species are considered to be highly detrimental owing to their potential to cause illness and dairy product spoilage (Faille et al., 2014). Moreover, being often associated with animal udders, these bacteria may easily spread through dairy production systems. The ability to form biofilm enables *Bacillus* species to thrive in the dairy-associated environment, as it facilitates their dispersion and survivability (Marchand et al., 2012; Shaheen et al., 2010). Furthermore, these bacteria can produce heat-resistant endospores which play an important role in bacterial persistence and biofilm establishment in the dairy environment (Ostrov et al., 2016). Members of *Bacillus* genus also possess swarming motility, which might facilitate microbial survival in the environment and surface colonization, leading to biofilm formation (Salvetti et al., 2011).

Biofilm formation by *Bacillus* species depends on the synthesis of an extracellular matrix which holds the constituent cells together. In *B. subtilis* the matrix has two main components, an exopolysaccharide (EPS) synthesized by the products of the *epsA-O* operon, and amyloid-like fibers encoded by *tasA* located in the *tapA-sipW-tasA* operon. Another extracellular polymer, γ-poly-DL-glutamic acid (PGA) is produced in copious amounts by some *B. subtilis* strains and can enhance the formation of submerged biofilms (Morikawa et al., 2006; Stanley and Lazazzera, 2005; Yu et al., 2016). Biosynthesis of PGA relies on the *pgsB-pgsC-pgsA-pgsE* operon (Yu et al., 2016).

It appears that biofilm formation by *Bacillus* is affected by environmental conditions (Pasvolsky et al., 2014; Shemesh and Chai, 2013). Being considered as a survival strategy, biofilm formation might enable adaptation of bacteria to certain environmental niches. Consequently, it is conceivable that biofilms formed by the strains, obtained from the dairy-associated environment, could differ from biofilms formed by the non-dairy strains. We therefore hypothesized that genotypic differences would explain an adaptability of certain *Bacillus* strains to the dairy-associated environment. Thus, we performed genomic and phenotypic comparison between non-dairy and dairy-associated *Bacillus* isolates in context of biofilm formation. The results of the study provide new insights into adaptation and persistence mechanisms of *Bacillus* species in the dairy environment.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Following bacterial strains were used in this study: (i) dairy-

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Table 1
General genomic features of the milk-associated *Bacillus* strains used in this study.

Isolate	Origin	The number of contigs	N_{50} of The contigs, bp	Average contig size	Estimated genome size, bp	GC content, %	Predicted genes	Protein-coding genes	Mobile element proteins	Phage-associated proteins	tRNA coding genes	rRNA coding genes
B. para- licheniformis S127	sheep milk (Ihud, Israel)	48	459,307	94,995.8	4,559,800	45.5	4923	4806	6	118	81	36
B. licheniformis MS310	sheep milk (Bet Zeid, Israel)	55	295,756	126,275.2	4,167,083	46.1	4583	4283	1	111	79	21
B. subtilis MS302	sheep milk (Bet Zeid, Israel)	33	300,361	75,058.7	4,128,231	44	4356	4257	5	50	85	14
B. para-licheniformis MS303	cow milk (Midrach-Oz, Israel)	37	581,698	116,691.2	4,317,578	45.9	4685	4588	0	117	79	18
B. licheniformis MS307	cow milk (Kibutz Nirim, Israel)	26	409,007	165,364.9	4,299,489	45.8	4764	4671	2	134	77	16

associated isolates, such as *B. paralicheniformis* S127 (Ostrov et al., 2015), *B. licheniformis* MS310, *B. subtilis* MS302, *B. paralicheniformis* MS303 and *B. licheniformis* MS307; (ii) non-dairy isolates: *B. paralicheniformis* ATCC8480 (ATCC strain of unknown origin), *B. subtilis* NCIB3610 (descendant of *B. subtilis* Marburg) and its mutant derivative *B. subtilis* YC295 (Δ*ywcC*; Yu et al., 2016). In addition, genome sequences of *B. subtilis* 168, *B. subtilis* subsp. *spizizenii* W23 and *B. licheniformis* ATCC14580 were used for genome comparison between the dairy- and non-dairy *Bacillus* isolates (Supplementary Tables 7 and 8).

The dairy-associated bacterial isolates were obtained from Israeli dairy farms (Table 1) according to the methods described by Parry et al. (1983), and identified as *Bacillus* species based on their morphology and their ability to form spores (Parkinson et al., 1999). The strains were kindly provided to us by the Laboratory for Udder Health and Milk Quality (Israel Dairy Board, Caesarea, Israel). For routine growth, the strains were propagated in Lysogeny broth (LB; 10 g tryptone, 5 g yeast extract, 5 g NaCl per liter, pH 7) or on solid LB medium supplemented with 1.5% agar at 37 °C. For biofilm generation, bacteria were cultivated in 5% skim milk (SM) (BD Difco, Sparks, MD, USA), which was prepared as described previously (Pasvolsky et al., 2014). For the proteolytic activity assay, the SM was supplemented with 1.5% agar. For the lipolytic activity assay, Spirit Blue agar (SBA; 10 g casein enzymatic hydrolysate, 5 g yeast extract, 0.15 g Spirit Blue, 17 g agar per liter, Himedia Laboratories, Mumbai, India) was used. The medium was sterilized by autoclaving at 121 °C for 15 min and supplemented with 30 ml of filter-sterilized lipase substrate (1 ml Tween 80 and 100 ml olive oil [Sigma Aldrich, Buchs, Switzerland], and 400 ml distilled water per 500 ml).

2.2. Whole-genome sequencing and bioinformatics analysis

Genomic DNA was extracted using GenElute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions and as described previously (Assaf et al., 2015). Whole-genome sequencing was performed as described earlier (Ostrov et al., 2015). The draft genome sequences of the strains were determined by *de novo* assembly of paired-end MiSeq Illumina sequence data (not compared with reference-based assembly). DNA was prepared for sequencing using the Nextera Library Preparation Kit (Epicentre, Madison, WI, USA). Assembly was achieved using the A5-miseq pipeline (Coil et al., 2015; Gurevich et al., 2013). Annotation was performed in RAST (Aziz et al., 2008) before being submitted to NCBI. *B. licheniformis* MS310, *B. subtilis* MS302, *B. paralicheniformis* MS303 and *B. licheniformis* MS307 whole-genome shotgun projects are deposited at DDBJ/EMBL/GenBank under accession numbers LFOC00000000, MIPQ00000000, MIZD00000000, MIZE00000000, and MIZF00000000, respectively.

Putative homologues of known *B. subtilis* biofilm genes were identified using RAST genome annotation and BLASTP. The percentage of amino acid identity between protein sequences of these putative homologues and the corresponding proteins from *B. subtilis* 168 (closely phylogenetically related to *B. subtilis* 3610) was determined using BLASTP. Genes encoding proteolytic/lipolytic enzymes and swarming motility determinants were identified using RAST genome annotation and BLASTP.

Additional putative biofilm-associated determinants were identified by analyzing and comparing genetic repertoire (all genes present in the genomes) of the tested strains using Proteinortho (proteinortho5 script; Lechner et al., 2011). Homologues of genes, present in strong-biofilm forming dairy *Bacillus* and missing in one/several non-dairy isolates, were selected for further analysis. Relevance of the selected genes to biofilm formation was determined using BLASTp comparison to protein sequence database and literature analysis.

A phylogenetic tree showing the relationships among the identified strains, based on gain/loss of the biofilm-related genes was constructed using a binary matrix containing 36 orthologous genes, putatively

associated with biofilm formation (Supplementary Tables 7 and 8). The presence/absence of the genes was determined by OrthoFinder software (Emms and Kelly, 2015) analysis. The tree was visualized using ape R package (Paradis et al., 2004).

Protein sequence alignment of selected genes was performed in Multalin (version 5.4.1.; Corpet, 1988).

2.3. Submerged biofilm formation

Submerged biofilms of the tested strains were generated in SM using a constant-depth film fermenter (CDFF; generated by the laboratory of Willson and Pratten; Feldman et al., 2017; Pratten, 2007). Briefly, the CDFF consists of a glass vessel with stainless-steel plates at the top and bottom (Supplementary Fig. 2). The top plate contains ports for supplying either bacterial culture or fresh medium, and a port for aeration and for sampling. The bottom plate provides an outlet for waste. The vessel houses a rotating stainless-steel disk (turntable, driven by a motor) with wells for sample deposition. Each well contains a polytetrafluoroethylene (PTFE) sampling pan with adjusted PTFE cylinders immersed (100–400 μm) in the body of the pan. Biofilm is generated on the PTFE cylinders. The most characteristic feature of the CDFF is the PTFE scraper blades, designed to restrict biofilm growth in height and spread bacterial culture or fresh medium across the turntable (Ludecke et al., 2014).

Overnight cultures of the tested strains (generated in LB at 23 °C, 90 rpm) were pumped into the CDFF for 5 h through the inoculum port; then growth medium (SM) was pumped into the fermenter through another port. Medium was supplied at 60 ml/h, and the rate of the turntable rotation was 2 rpm. Biofilms generated on PTFE cylinders, following 18 h of incubation at 30 °C, were washed with sterile distilled water (to remove unattached cells) and stained using a the FilmTracer™ LIVE/DEAD Biofilm Viability Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's protocol. Stained samples were visualized by confocal laser scanning microscopy (CLSM) (Olympus IX81, Japan) at 50 μm scale.

Biofilm depth values for the tested samples were based on the data obtained using CLSM. The Image J program (National Institutes of Health, Bethesda, MD, USA) was used to analyze the relative quantities of live and dead cells in the biofilm, by calculating the fluorescence intensity per area for each color (green for live cells, red for dead cells) separately (Assaf et al., 2015). The measured area of all images was the same throughout the experiment.

To determine the number of viable cells, attached biofilm cells were mechanically removed (by scrubbing the surfaces of PTFE plugs, exposed to biofilm cells) into 1 ml of phosphate buffer saline (PBS, Sigma Aldrich, USA). Next, biofilm cells were separated using sonication (Sonics Vibra cell; amplitude 70%, pulse 10 s, pause 10 s, duration 2 min). Serial 10-fold dilutions of each sample were performed followed by plating out three appropriate dilutions on LB agar plates for CFU analysis. For each tested bacterial strain, two different biological experimental repeats were performed; each repeat was carried out in triplicate.

2.4. Biofilm bundle formation

For analysis of biofilm bundle formation, first the strains were grown overnight in LB at 23 °C, on a rotary shaker at 90 rpm. Cells from the starter cultures were seeded (1:100 dilution) in Erlenmeyer flasks with SM and incubated at 30 °C, 25 rpm for 24 h. Aliquots (4 ml) of each culture were collected and centrifuged at 12,000 rpm for 2 min. The supernatant was decanted; cells were washed once with PBS, and stained using the LIVE/DEAD staining method. Next, the samples were washed and resuspended in 150 μl PBS. A 5-μl aliquot of each sample was placed on a glass slide and visualized by CLSM at 50 μm scale. Image J program was used to determine the relative quantities of live and dead cells in the biofilm (Assaf et al., 2015). For each tested bacterial strain, two different biological experimental repeats were performed; each repeat was carried out in triplicate.

2.5. Pellicle formation

For analysis of pellicle formation, overnight starter cultures of the tested strains were seeded (1:100 dilution) into glass bottles (total volume 50 ml) containing 20 ml SM. The bottles were incubated at 30 °C, 25 rpm for 48 h. For microscopic visualization of the formed pellicles, sterile glass slides (24 × 60 mm) were inserted into each bottle before the incubation. Then, the slides with the attached pellicle fragments were removed from the bottles, washed once with PBS, and stained using the LIVE/DEAD staining method. Pellicles were visualized by CLSM at 50 μm scale. Image J program was used for analysis of the relative quantities of live and dead cells in pellicles (Assaf et al., 2015). For each tested bacterial strain, two different biological experimental repeats were performed; each repeat was carried out in triplicate.

2.6. Lipolytic activity assay

Cells were grown in LB at 37 °C, 150 rpm for 5 h (to the beginning of the stationary phase), resuspended to an optical density at 600 nm (OD_{600}) of 1 in LB and seeded on SBA supplemented with lipase substrate. The samples were incubated at 30 °C for 72 h. Lipolytic activity was determined according to the change of color of SBA (from blue to yellow) around bacterial colonies (Abdou, 2003). For each tested bacterial strain, two different biological experimental repeats were performed; each repeat was carried out in triplicate.

2.7. Proteolytic activity assay

Cells were grown in LB at 37 °C, 150 rpm for 5 h, resuspended to OD_{600} = 1 in LB and seeded on SM supplemented with 1.5% agar. The samples were incubated at 30 °C for 72 h. Proteolytic activity was determined according to the change in color of SM (from white to transparent) around bacterial colonies (Kumari and Sarkar, 2014). For each tested bacterial strain, two different biological experimental repeats were performed; each repeat was carried out in triplicate.

2.8. Swarm expansion assay

To determine swarming motility rates, cells were grown in LB at 37 °C, 150 rpm for 5 h, resuspended to OD_{600} = 1 in LB, seeded on freshly prepared LB plates containing 0.5% agar, and incubated at 37 °C for 5 h. A mark was drawn on the bottom (outside surface) of the Petri plate to demark the colony origin. Swarm rates were determined by measuring the distance from the colony origin to the swarm front as a function of time (Kearns and Losick, 2003). For each tested bacterial strain, two different biological experimental repeats were performed; each repeat was carried out in triplicate.

3. Results

3.1. Identification of genomic features of dairy-associated Bacillus isolates

We started this investigation with identification and sequencing of new milk isolates of strong biofilm-forming *Bacillus* species obtained from Israeli dairy farms. The isolates were defined as *B. licheniformis* MS310, *B. subtilis* MS302, *B. paralicheniformis* MS303 and *B. licheniformis* MS307. We have also sequenced a non-dairy isolate *B. paralicheniformis* 8480 (previously identified as *B. licheniformis* 8480; Madslien et al., 2013), which was used for genomic and phenotypic comparison to the newly-identified dairy *Bacillus*.

A summary of the genomic features of the dairy-associated *Bacillus* isolates (including previously identified dairy-associated *B. paralicheniformis* S127; Ostrov et al., 2015), *B. paralicheniformis* 8480 and

several previously identified non-dairy *Bacillus* (*B. subtilis* 168, 3610, *B. subtilis* subsp. *spizizenii* W23 and *B. licheniformis* 14580) is provided in Table 1 and Supplementary Table 1. A genome size (4.1–4.6 Mb), G + C content (44–46.1%) and number of predicted genes (4356–4923) did not differ significantly among the newly sequenced and previously identified *Bacillus* strains (Kunst et al., 1997; Veith et al., 2004; Zeigler, 2011). The number of protein-encoding genes, predicted for the isolates using RAST (Aziz et al., 2008), ranged from 4257 to 4806. All newly-sequenced strains, except *B. paralicheniformis* MS303 and *B. paralicheniformis* 8480, had genes encoding mobile genetic elements. The number of phage-associated proteins ranged from 50 for *B. subtilis* MS302 to 134 for *B. licheniformis* MS307. Estimated numbers of rRNA- (16–36) and tRNA- (77–86) encoding genes did not differ markedly between the newly sequenced and other *Bacillus* strains (Kunst et al., 1997; Veith et al., 2004; Zeigler, 2011).

3.2. Proteolytic and lypolitic capability of the dairy-associated Bacillus isolates

Firstly, we determined the ability of the tested bacterial isolates to utilize milk constituents such as proteins and fats. According to our data, all tested strains were found to be proteolytic; *B. licheniformis* MS310 performed proteolysis most efficiently (Supplementary Table 2). To elucidate whether the differences in proteolytic activity of the tested *Bacillus* result from genetic differences, we screened for the presence of genes encoding proteolytic enzymes in the genomes of the dairy-associated *Bacillus* isolates, *B. subtilis* 3610 and *B. paralicheniformis* 8480. According to our analysis, genomes of all tested strains contained genes encoding proteases, peptidases and peptide transporters (Liu et al., 2010; Switt et al., 2014). However, we did not observe correlation between copy number of genes encoding proteolytic enzymes in the tested bacteria and their proteolytic efficiency (Supplementary Table 2).

We also analyzed the ability of the dairy-associated *Bacillus* isolates, *B. subtilis* 3610 and *B. paralicheniformis* 8480 to perform lipolysis. According to our data, all tested strains were lipolytic (Supplementary Fig. 1). Genomic analysis has revealed that all tested strains contained fairly similar repertoire of genes encoding lipolytic enzymes (such as lipases, phospholipases and esterases (Arpigny and Jaeger, 1999; Supplementary Table 2).

3.3. Swarming motility of the dairy-associated Bacillus isolates

Next, we characterized the capacity of the milk isolates for swarming motility. Swarm expansion assay was employed to quantify this motility (Kearns and Losick, 2003). *B. licheniformis* MS310 showed the highest swarming rates, while swarm expansion in *B. paralicheniformis* 8480 could not be detected during 5 h of observation (Fig. 1). According to our data, the tested bacterial isolates contained genes related to swarming behavior (Supplementary Table 3) such as a flagellin-encoding gene, chemotaxis response and flagellar rotation determinants (Kearns and Losick, 2003), transcriptional factor SigD, surfactin synthethases, swarming motility proteins SwrA (which also takes part in submerged biofilm formation; Kearns et al., 2004; McLoon et al., 2011), SwrB and SwrC (Kearns et al., 2004). Notably, we could not identify homologues of surfactin synthethase SrfAA (Kearns et al., 2004) in the genomes of *B. subtilis* MS302 and *B. licheniformis* MS307; and the homologue of SrfAB in the genome of *B. paralicheniformis* MS303 (Supplementary Table 3).

3.4. Dairy-associated Bacillus strains form robust biofilms during growth in milk

Since biofilm formation can potentially play a major role in bacterial survival in the dairy industry (Shaheen et al., 2010; Marchand et al., 2012), we evaluated the ability of the dairy isolates to form

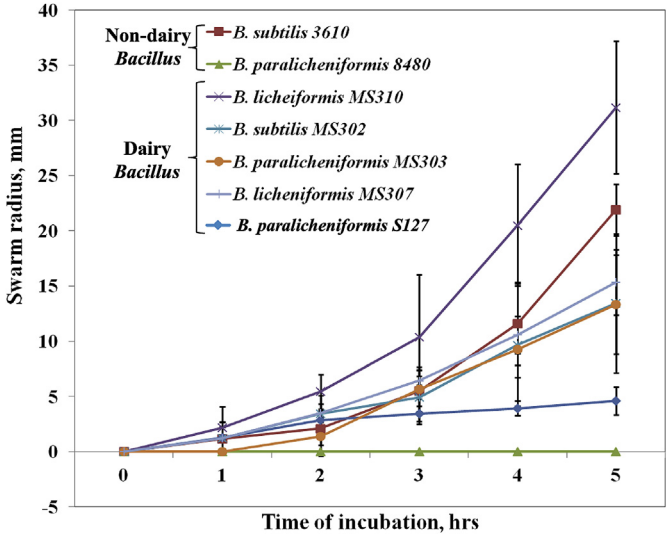

Fig. 1. Swarming motility rates of the tested strains.

biofilm in milk. Biofilm development generally occurs on dairy equipment surfaces that are in contact with milk (Flint et al., 1997; Sharma and Anand, 2002; Shaheen et al., 2010); we therefore evaluated the ability of the tested *Bacillus* isolates to form submerged surface-associated biofilm. We used a CDFF (Supplementary Fig. 2) as a model for the generation of submerged biofilm as it provides a tightly controlled environment for biofilm development and maintenance (Ludecke et al., 2014). The z (depth) restriction of the cultivated biofilms was adjusted to 100, 200, 300 or 400 μm. To evaluate submerged biofilm formation by the dairy *Bacillus* isolates, non-dairy isolates *B. subtilis* 3610 and *B. paralicheniformis* 8480 were a used as a reference. After 18 h of incubation in the CDFF, all tested strains except *B. subtilis* 3610 and *B. paralicheniformis* 8480 formed robust submerged biofilm in SM (Fig. 2, Supplementary Table 4).

According to our previous study (Pasvolsky et al., 2014), *Bacillus* strains form biofilm-related structures termed bundles during their growth in milk, a phenomenon that is conserved in *Bacillus* species. Since biofilm bundles can be viewed as floating biofilms, their existence in milk may have highly undesirable implications. Therefore, we determined the ability of the isolates to form this type of biofilm using the method described by Pasvolsky et al. (2014). To assess bundle formation by the tested strains, *B. subtilis* 3610 and *B. paralicheniformis* 8480 (previously shown to form bundles in SM) were used as a reference. As seen in Supplementary Fig. 3, all dairy *Bacillus* isolates formed biofilm bundles in SM. Among the tested isolates, *B. subtilis* MS302, *B. paralicheniformis* MS303, *B. licheniformis* MS307 and especially *B. licheniformis* MS310 produced significantly higher numbers of bundles (per microscope field of view, Supplementary Table 5) which contained notably higher quantities of bundled cells (according to fluorescence intensity measurement, Supplementary Table 5) compared to 3610 and 8480.

Additionally, milk-associated *Bacillus* strains formed robust biofilm at the air–liquid interface (pellicles) in milk (Fig. 3, Supplementary Table 6). Interestingly, the formation of pellicle (which can be also viewed as floating biofilm) in milk was not observed for *B. subtilis* 3610, or *B. paralicheniformis* 8480 (Fig. 3).

3.5. Genome comparison in context of biofilm formation between dairy-associated and non-dairy Bacillus strains

Since dairy-associated *Bacillus* strains are characterized with robust biofilm formation, we decided to identify putative biofilm-associated genes in the genomes of these strains and compared them to non-dairy *Bacillus* isolates. For our analysis, we selected the following non-dairy

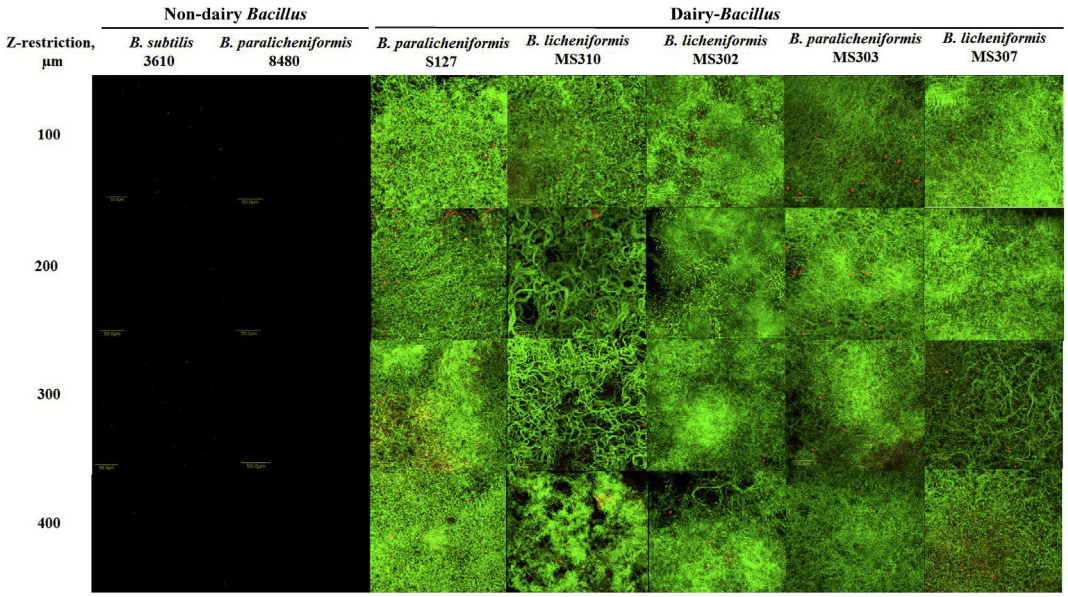

Fig. 2. Submerged biofilm formation by the *Bacillus* strains in SM. Live cells stained green, dead cells stained red. Stained samples were visualized by CSLM at 50 μm scale. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

associated strains: *B. subtilis* 3610, *B. subtilis* 168 (model strain used in *B. subtilis* research, domesticated and biofilm-compromised; Zeigler et al., 2008), *B. subtilis* subsp. *spizizenii* W23 (viewed as a 'wild-type' counterpart to strain 168; Zeigler, 2011), *B. paralicheniformis* 8480, *B. licheniformis* 14580 (soil isolate, type strain used in *B. licheniformis* research; Veith et al., 2004). According to our results, the homologues of following genes were identified for the tested strains: (i) encoding biofilm matrix components (genes of *epsA-O, tapA-sipW-tasA, pgsBCEA* operons, etc; Vlamakis et al., 2013); (ii) regulators of biofilm formation (sensor histidine kinases KinA – D, DegS; regulatory proteins Spo0A, SinI, SinR, AbrB, SlrR, SlrA, DegU, SwrA, CodY, RNA polymerase sigma factor RpoN; Chen et al., 2012; Hayrapetyan et al., 2015; Lindback et al., 2012; Vlamakis et al., 2013); (iii) quorum sensing determinants ComP, ComQ, Sfp (Chen et al., 2012; Lopez et al., 2009; Vlamakis et al., 2013; Supplementary Table 7). Similarity of the predicted products of these genes between *B. subtilis* 168 (taken as reference) and other *Bacillus* strains varied: from highly conserved in non-dairy isolate *B. subtilis* 3610, to less conserved in *B. subtilis* subsp. *spizizenii* W23 and *B. subtilis* MS302, and found to be most dissimilar in *B. paralicheniformis* and *B. licheniformis* strains (Supplementary Table 7). According to our analysis, protein sequences of certain regulatory proteins such as master regulator Spo0A, major repressors of *epsA-O* and *tapA-sipW-tasA* operons – SinR and AbrB, CodY, DegS-DegU two-component system and PGA biosynthesis operon *pgsBCAE* (Vlamakis et al., 2013) were found as highly conserved in all tested strains (Supplementary Table 7). However, other regulatory genes as well as genes of *epsA-O* and *tapA-sipW-tasA* operons, protein tyrosine-phosphatase YwqE and PGA hydrolase PdgS, were considerably less conserved between the tested strains (Supplementary Table 7). Furthermore, significant sequence dissimilarity between non-dairy isolate *B. licheniformis* 14580 and other tested strains was observed for paralogous SinR-antirepressor SlrA (activates expression of biofilm matrix operons; Vlamakis et al., 2013,

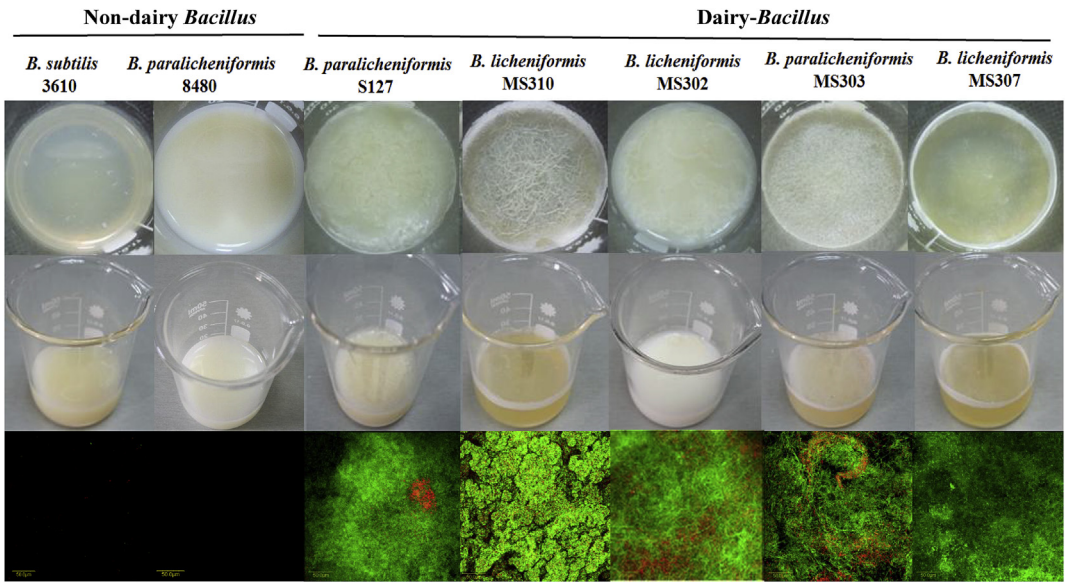

Fig. 3. Pellicle formation by the tested *Bacillus* strains in SM. Live cells stained green, dead cells stained red. Stained samples were visualized by CSLM at 50 μm scale. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Supplementary Table 7B, Supplementary Fig. 4). Drastic sequence dissimilarity between *B. subtilis* 168, 3610 and other tested strains was also observed for quorum sensing determinants ComP, ComQ, and Sfp (dysfunctional in strain 168 due to point mutation, Kearns et al., 2004; Supplementary Table 7B). Importantly, we could not identify homologue of sensor histidine kinase ComP in the genome of *B. licheniformis* 14580. Also, a heterogeneity in sequence similarity between *B. subtilis* 168, 3610, and other tested strains was observed for exopolysaccharide biosynthesis enzyme YpqP (disrupted by Spβ phage in strains 168, 3610; Sanchez-Vizuete et al., 2015; Supplementary Table 7A, Supplementary Fig. 4).

We further hypothesized that there can be additional biofilm-related genes in the genomes of the dairy *Bacillus* isolates (compared to non-dairy-associated *Bacillus* strains), which might lead to robust biofilm phenotype. To test our assumption, we compared homologues of all genes present in the genomes of the tested dairy-associated and non-dairy *Bacillus* using Orthoscript (Lechner et al., 2011). Following the performed analysis, we identified 68 genes present predominantly in the genomes of strong biofilm-forming dairy *Bacillus* (absent in the genomes of one or several non-dairy *Bacillus* isolates; Supplementary Fig. 7). Importantly, significant number of genes (at least 29), identified mainly in the genomes of strong biofilm-forming dairy *Bacillus* isolates, can be associated with biofilm formation according to literature analysis (Supplementary Table 8). We used these genes, in addition to previously known biofilm determinants (which were found as most differentially annotated in dairy vs. non-dairy isolates; Supplementary Table 7), for the construction of a phylogenetic tree based on presence/absence of the putative biofilm-associated genes (Fig. 4). According to our results, the generated tree displayed distribution of the following groups of strains: i) dairy-associated *B. licheniformis, B. paralicheniformis* and *B. subtilis* MS302; ii) non-dairy *B. subtilis* W23, 3610 and 168; iii) non-dairy *B. paralicheniformis* 8480; iv) non-dairy *B. licheniformis* 14580 (Fig. 4).

4. Discussion

The key finding of this study is related to the biofilm-forming ability of the *Bacillus* isolates in milk. Milk-holding equipment was previously considered to have two distinct but connected phases, available for microbial growth: the liquid phase, in which planktonic cells proliferate, and the solid–liquid interface where cells can attach and form biofilms (Somers et al., 2001; Marchand et al., 2012). However, we showed that dairy *Bacillus* isolates could form biofilm in both phases, mentioned above, and also in air–liquid interface. According to our results, the dairy-associated *Bacillus* strains formed robust surface-associated (submerged) biofilm in milk; whereas no notable submerged-biofilm formation was observed by non-dairy *B. paralicheniformis* 8480 or *B. subtilis* 3610. Unlike previous studies that used microtiter plates (Cherif-Antar et al., 2016; Zain et al., 2016) or stainless-steel coupons (Kumari and Sarkar, 2014; Zain et al., 2017) for submerged biofilm generation in milk, we used a CDFF – a continuous flow system that more closely simulates industrial conditions (e.g., the flow of liquid in the dairy equipment).

Apart from surface-associated biofilm, the dairy *Bacillus* isolates successfully formed other biofilm types in milk – bundles in the liquid phase and pellicles at the air–liquid interface. Importantly, pellicle formation in milk was observed only for the dairy-associated *Bacillus* strains, and not in the non-dairy isolates *B. subtilis* 3610 or *B. paralicheniformis* 8480. These results suggest that biofilm formation in the liquid phase and at the air–liquid interface by the dairy *Bacillus* isolates can serve as an adaptation to the conditions of the dairy environment. To this end, pellicle biofilm as well as biofilm bundles might readily develop in industrial storage and piping systems where the flow is moderate during operation or where residual liquid remains after a production cycle.

Taken together, the tested dairy isolates could display several modes of biofilm formation in milk, depending on environmental conditions. Robust biofilm formation by *Bacillus* strains might have highly undesirable implications for the dairy industry. Thus, biofilm might be a source of further contamination by disseminating vegetative cells, spores, or detached biofilm clumps that adhere to the dairy equipment components and lead to product contamination. Bundles or biofilm fragments might attach to the surface of the dairy equipment, or circulate through the milking pipelines, facilitating biofilm dispersal throughout the dairy processing equipment.

The distinctions in biofilm phenotype in milk between the dairy and non-dairy *Bacillus* isolates could be explained by differences in genes, associated with biofilm formation. According to our results, sequence dissimilarity between putative biofilm determinants (involved in biofilm formation in *B. subtilis* model strains) strongly correlated with observed biofilm phenotype. Furthermore, we could not identify homologue of transcription repressor YwcC in strong biofilm-forming dairy isolate *B. subtilis* MS302 as well as in all tested *B. paralicheniformis* and *B. licheniformis* strains. YwcC negatively regulates PGA and matrix

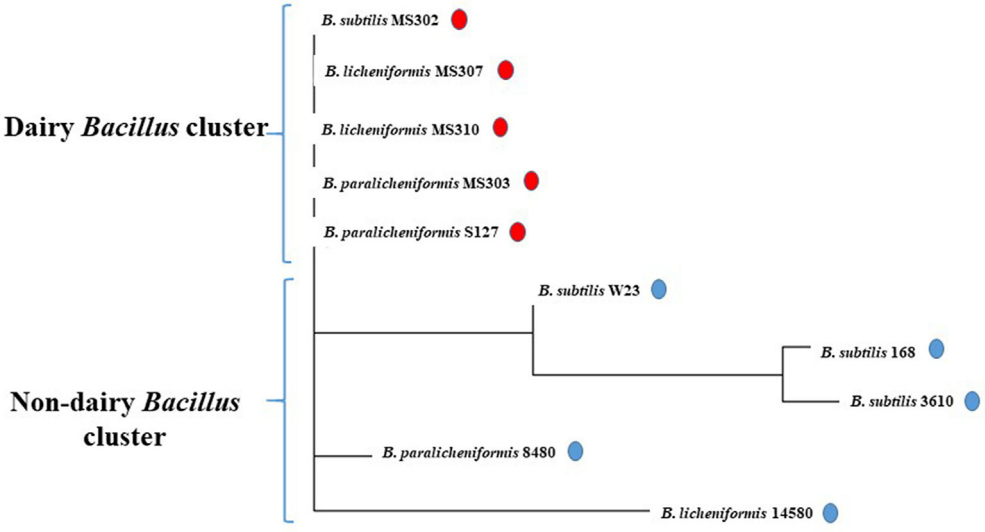

Fig. 4. Phylogenetic relationship of the identified *Bacillus* strains based on presence/absence of putative biofilm-associated genes (present in all dairy-associated *Bacillus* strains). The genes, used for the analysis are presented in the Supplementary Table 7 (indicated by asterisk) and 8.

genes (Yu et al., 2016); therefore, absence of this repressor leads to PGA overproduction (Yu et al., 2016), which could result in stronger biofilm formation. To this end, the *ΔywcC* mutant of *B. subtilis* could form notable pellicle in milk (Supplementary Fig. 5).

Notably, protein sequence of SinR antirepressor SlrA (activates matrix genes expression, negatively regulated by YwcC; Vlamakis et al., 2013) was observed as truncated in non-dairy *B. licheniformis* 14580 compared to the dairy isolates, which might be a result of mutation or phage disruption. In addition, we found that non-dairy *B. subtilis* (unable to form submerged biofilm/pellicle in milk) had a phage disruption of exopolysaccharide biosynthesis gene *ypqP*, which results in impaired submerged biofilm formation (Sanchez-Vizuete et al., 2015). Remarkably, *B. subtilis* MS302, characterized by robust submerged biofilm phenotype, had a *ypqP* sequence, highly similar to the non-disrupted *ypqP* of the *B. subtilis* NDmed isolate (able to form confluent submerged biofilm; Sanchez-Vizuete et al., 2015; Supplementary Fig. 6). Thus, our analysis revealed that the observed differences in biofilm phenotype between dairy and non-dairy *Bacillus* isolates can be connected to genes, regulating PGA and matrix genes production.

Furthermore, genome comparison analysis of the tested strains has revealed 68 additional genes, among which at least 29 can be associated with robust biofilm formation in the dairy *Bacillus* according to literature analysis (absent in one or several weak biofilm forming non-dairy isolates). The identified genetic determinants include transporters, genes governing carbon metabolism and fermentation, sugar metabolism and exopolysaccharide synthesis, fatty acids synthesis, flagellar motility, transcriptional regulators and ribosomal proteins. According to previous investigations, transport proteins can function as importers of molecules that influence biofilm formation (Auger et al., 2006; Hayrapetyan et al., 2015; Garai et al., 2017) or as exporters of biofilm components and pheromones (Heinrich et al., 2018). Notably, many of the identified genes are involved in the transport of glycerol, iron, branched-chain amino acids, sucrose, mannitol, celobiose, which are known to induce biofilm formation in *Bacillus* and other bacteria (Belitsky, 2015; Dogsa et al., 2013; Hayrapetyan et al., 2015a,b; Wu et al., 2012; Ymele-Leki et al., 2013). Presence of 2,3-butanediol dehydrogenase (S-alcohol forming, (R)-acetoin-specific)/acetoin (diacetyl) reductase in strong biofilm-forming dairy *Bacillus* can result in significant accumulation of small fermentation products, such as acetoin and 2,3-butanediol. These fermentation products, together with ethanol, acetate and lactate, trigger the biofilm pathway presumably through altered metabolism activities (Chen et al., 2015; Yan et al., 2017). Furthermore, non-dairy *B. subtilis* 3610 and 168 lack homologues of delta-acyl-lipid desaturase DesA. This enzyme participates in fatty acid biosynthesis, which is important for biofilm formation and sporulation in *B. subtilis* (Pedrido et al., 2013). In addition, non-dairy *Bacillus* isolates were lacking certain sporulation and spore germination proteins. The requirement of these genes for biofilm formation can be connected to Spo0A signaling network, which links between sporulation and biofilm formation (Fujita et al., 2005). This network may be so profoundly disrupted by the failure of these genes, that the biofilm branch of the Spo0A network is disrupted as well (Okshevsky et al., 2018). Moreover, biofilm formation from germinated spores is frequently observed in the dairy industry (Lindsay et al., 2005; Wijman et al., 2007). Also, non-dairy *Bacillus,* unable to form submerged biofilm and/or pellicle in milk, lacked homologues of flagellar-associated proteins (flagellar protein FlbD in *B. subtilis* 168; flagellar basal-body rod protein FlgB in *B. paralicheniformis* 8480). This observations correlate with previous studies (Houry et al. 2018; Okshevsky et al., 2018), indicating that flagella-mediated motility is important for static biofilm and pellicle formation in *B. cereus*.

Importantly, the results of genome comparison were supported by phylogenetic analysis based on the examined biofilm-related genes (both previously known and identified in this study). Thus, the clustering of the tested strains in accordance with the repertoire of putative biofilm determinants significantly resembles their grouping by the dairy/non-dairy origin and milk-associated biofilm phenotype. According to our results, such grouping is attributed mostly to gain/loss of putative biofilm-related genes, as well as mutations/phage disruptions in non-dairy *Bacillus* and low sequence similarity of certain genes between the dairy and non-dairy *Bacillus* isolates. These phenomena are, likely, the result of niche adaptation by the dairy-associated *Bacillus* strains. To this end, close phylogenetic relationship between the dairy *B. licheniformis*, *B. paralicheniformis* and *B. subtilis* MS302 strains, isolated from different geographical areas indicates that cognate forces may drive biofilm adaptation in the various sites of the dairy-associated environment.

An additional detrimental effect of biofilm formation by dairy-associated *Bacillus* is contamination of dairy products by enzymes such as proteases and lipases (Teh et al., 2012, 2013). Lipolysis and proteolysis have been shown to be considerably higher within biofilms than in the corresponding planktonic cultures (Teh et al., 2012). Moreover, the accumulation of enzymes in the biofilm may facilitate bacterial survival in the dairy environment (Teh et al., 2013). According to our results, all tested strains (including non-dairy isolates 3610 and 8480) performed lipolysis efficiently and contained fairly similar repertoire of lipolysis-associated genes. Therefore, based on our analyses we could not define a clear link between lipolytic activity and biofilm formation by the tested strains.

All tested strains (including non-dairy isolates *B. subtilis* 3610 and *B. paralicheniformis* 8480) performed proteolysis efficiently, with *B. licheniformis* MS310 having the highest proteolytic activity. However, all tested strains, including MS310, 3610 and 8480, contained similar repertoire of genes encoding proteolytic enzymes; likewise there was no obvious correlation between copy number of proteolysis-associated genes in the examined bacteria and their degree of proteolytic activity. This can be explained by either differences in regulation of proteolytic genes expression in the tested bacterial isolates or presence of yet uncharacterized proteolysis-associated genes. Importantly, highly proteolytic strain *B. licheniformis* MS310 has significantly stronger pellicle and bundle biofilm formation of all of the isolates. To this end, Yoo et al. (2006) showed that milk proteins are a good substrate for bacterial growth and proliferation. Therefore, we further speculate that the ability to perform proteolysis might facilitate bacterial survival in the dairy-associated environment.

In addition, we analyzed the isolates' capacity for swarming motility as this might confer an important advantage due to the availability of new nutrients (Shemesh et al., 2014). Swarming motility was observed with all tested dairy isolates; *B. licheniformis* MS310 had the highest swarming rates. Importantly, we did not observe notable differences in genes, governing swarming motility between the tested strains; except the absence of surfactin synthetase SrfAA in the genomes of *B. subtilis* MS302 and *B. licheniformis* MS307; and the homologue of SrfAB in the genome of *B. paralicheniformis* MS303 (which did not abolish or significantly reduce swarming motility rates compared to other tested strains). We speculate that swarming motility in tested strains might serve as a strategy for surface colonization and expansive growth during food processing (Shemesh et al., 2014). Moreover, swarming motility often precedes biofilm formation (Verstraeten et al., 2008; Hamouche et al., 2017) and ultimately determines where the biofilm will form (Hamouche et al., 2017).

In conclusion, the results of this study indicate that milk-associated *Bacillus* strains are characterized by formation of robust biofilm in milk, which was not observed for the tested non-dairy *Bacillus* isolates. Moreover, the differences in observed biofilm phenotypes strongly correlate with the presence or absence of putative biofilm-associated determinants in the genome. Therefore, we believe that biofilm formation can be a presumable adaptation strategy of *Bacillus* strains to the dairy environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.02.015.

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