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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

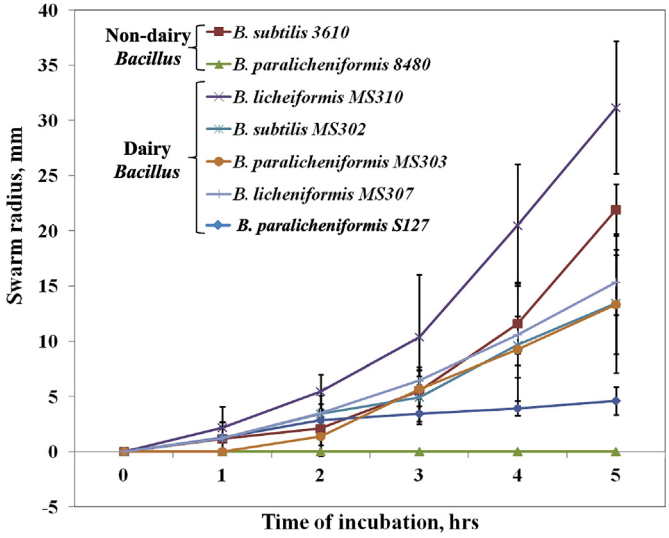

Fig. 1. Swarming motility rates of the tested strains.

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

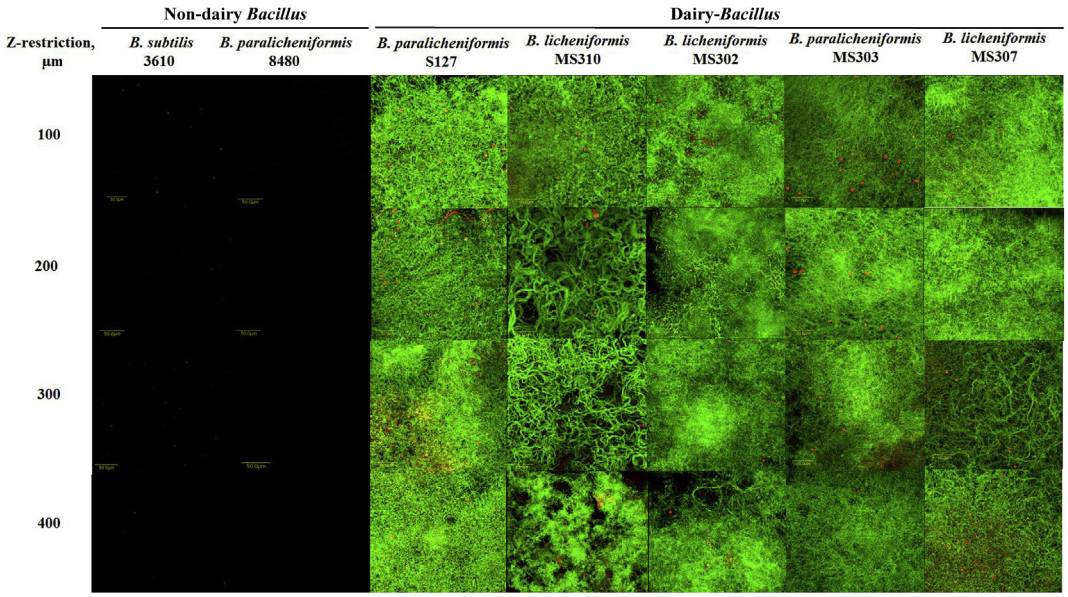

Fig. 2. Submerged biofilm formation by the *Bacillus* strains in SM. Live cells stained green, dead cells stained red. Stained samples were visualized by CSLM at 50 μm scale. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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,

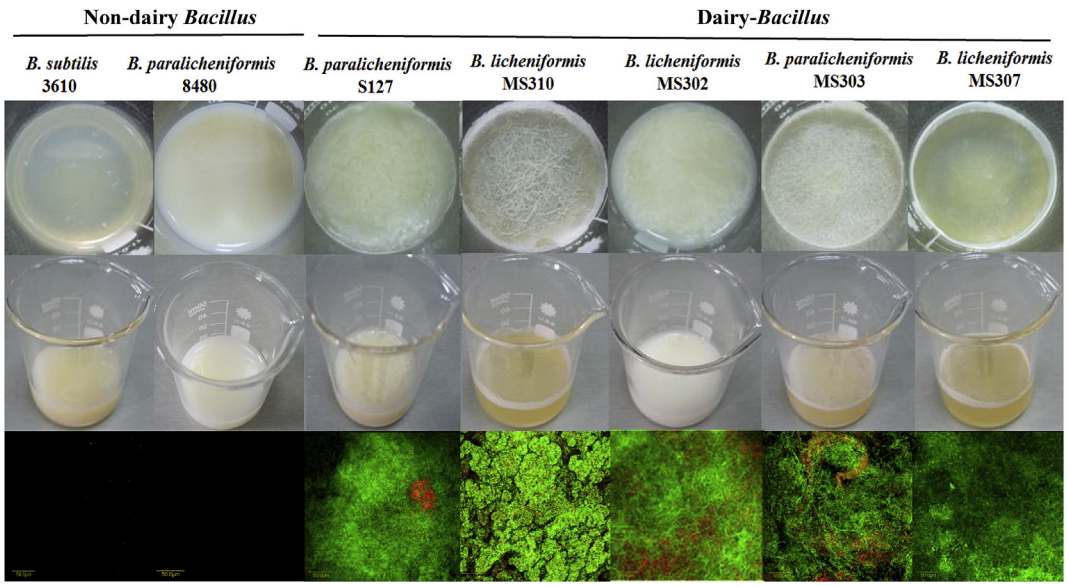

Fig. 3. Pellicle formation by the tested *Bacillus* strains in SM. Live cells stained green, dead cells stained red. Stained samples were visualized by CSLM at 50 μm scale. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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

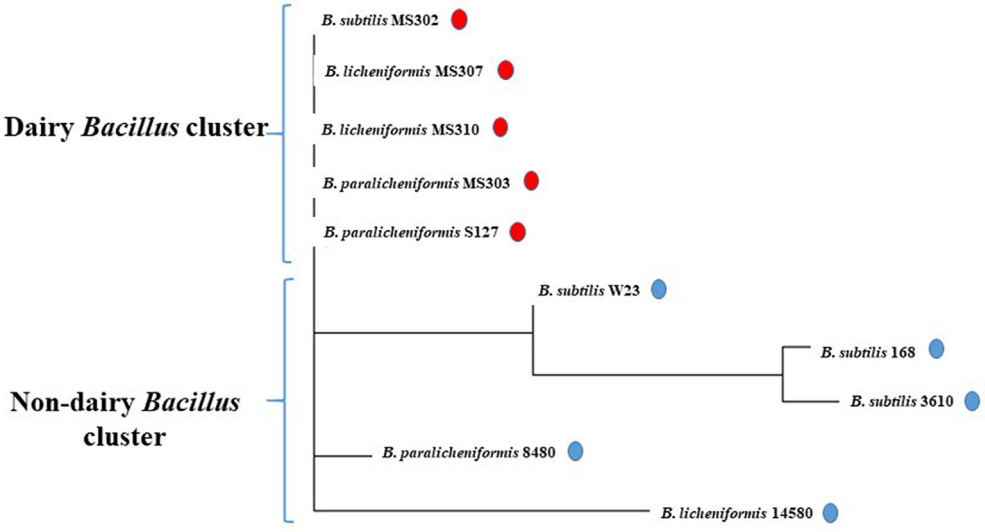

Fig. 4. Phylogenetic relationship of the identified *Bacillus* strains based on presence/absence of putative biofilm-associated genes (present in all dairy-associated *Bacillus* strains). The genes, used for the analysis are presented in the Supplementary Table 7 (indicated by asterisk) and 8.

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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References

Abdou, A.M., 2003. Purification and partial characterization of psychrotrophic *Serratia marcescens* lipase. J. Dairy Sci. 86, 127–132.
Arpigny, J.L., Jaeger, K.E., 1999. Bacterial lipolytic enzymes: classification and properties. Biochem. J. 343, 177–183.
Assaf, D., Steinberg, D., Shemesh, M., 2015. Lactose triggers biofilm formation by *Streptococcus mutans*. Int. Dairy J. 42, 51–57.
Auger, S., Krin, E., Aymerich, S., Gohar, M., 2006. Autoinducer 2 affects biofilm formation by *Bacillus cereus*. Appl. Environ. Microbiol. 72, 937–941.
Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O., 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics 9.
Belitsky, B.R., 2015. Role of branched-chain amino acid transport in *Bacillus subtilis* CodY activity. J. Bacteriol. 197, 1330–1338.
Chen, Y., Chai, Y.R., Gu, J.H., Losick, R., 2012. Evidence for cyclic Di-GMP-mediated signaling in *Bacillus subtilis*. J. Bacteriol. 194, 5080–5090.
Chen, Y., Gozzi, K., Yan, F., Chai, Y.R., 2015. Acetic acid acts as a volatile signal to stimulate bacterial biofilm formation. mBio 6.
Cherif-Antar, A., Moussa-Boudjemaa, B., Didouh, N., Medjahdi, K., Mayo, B., Florez, A.B., 2016. Diversity and biofilm-forming capability of bacteria recovered from stainless steel pipes of a milk-processing dairy plant. Dairy Sci. Technol. 96, 27–38.
Coil, D., Jospin, G., Darling, A.E., 2015. A5-miseq: an updated pipeline to assemble microbial genomes from Illumina MiSeq data. Bioinformatics 31, 587–589.
Corpet, F., 1988. Multiple sequence alignment with hierarchical-clustering. Nucleic Acids Res. 16, 10881–10890.
Dogsa, I., Brloznik, M., Stopar, D., Mandic-Mulec, I., 2013. Exopolymer diversity and the role of levan in *Bacillus subtilis* biofilms. PLoS One 8.
Emms, D.M., Kelly, S., 2015. OrthoFinder: solving fundamental biases in whole genome comparisons dramatically improves orthogroup inference accuracy. Genome Biol. 16.
Faille, C., Benezech, T., Midelet-Bourdin, G., Lequette, Y., Clarisse, M., Ronse, G., Ronse, A., Slomianny, C., 2014. Sporulation of *Bacillus* spp. within biofilms: a potential source of contamination in food processing environments. Food Microbiol. 40, 64–74.
Feldman, M., Shenderovich, J., Lavy, E., Friedman, M., Steinberg, D., 2017. A sustained-release membrane of thiazolidinedione-8: effect on formation of a *Candida*/bacteria mixed biofilm on hydroxyapatite in a continuous flow model. BioMed Res. Int. 2017, 3510124.
Flint, S.H., Bremer, P.J., Brooks, J.D., 1997. Biofilms in dairy manufacturing plant description, current concerns and methods of control. Biofoul. J. Bioadhes. Biofilm Res. 11 (1), 81–97. https://doi.org/10.1080/08927019709378321.
Fujita, M., Gonza, E., Losick, R., 2005. High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis*. J. Bacteriol. 187, 1357–1368.
Garai, P., Chandra, K., Chakravortty, D., 2017. Bacterial peptide transporters: messengers of nutrition to virulence. Virulence 8, 297–309.
Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G., 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29, 1072–1075.
Hamouche, L., Laalami, S., Daerr, A., Song, S., Holland, I.B., Seror, S.J., Hamze, K., Putzer, H., 2017. *Bacillus subtilis* swarmer cells lead the swarm, multiply, and generate a trail of quiescent descendants. mBio 8, e02102–e02116.
Hayrapetyan, H., Muller, L., Tempelaars, M., Abee, T., Groot, M.N., 2015a. Comparative analysis of biofilm formation by *Bacillus cereus* reference strains and undomesticated food isolates and the effect of free iron. Int. J. Food Microbiol. 200, 72–79.
Hayrapetyan, H., Tempelaars, M., Groot, M.N., Abee, T., 2015b. *Bacillus cereus* ATCC 14579 RpoN (Sigma 54) is a pleiotropic regulator of growth, carbohydrate metabolism, motility, biofilm formation and toxin production. PLoS One 10.
Heinrich, J., Lunde, T., Kontinen, V.P., Wiegert, T., 2018. The *Bacillus subtilis* ABC transporter EcsAB influences intramembrane proteolysis through RasP. Microbiology 154, 1989–1997.
Houry, A., Briandet, R., Aymerich, S., Gohar, M., 2018. Involvement of motility and flagella in *Bacillus cereus* biofilm formation. Microbiology 156, 1009–1018.
Kearns, D.B., Chu, F., Rudner, R., Losick, R., 2004. Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. Mol. Microbiol. 52, 357–369.
Kearns, D.B., Losick, R., 2003. Swarming motility in undomesticated *Bacillus subtilis*. Mol. Microbiol. 49, 581–590.
Kumari, S., Sarkar, P.K., 2014. In vitro model study for biofilm formation by *Bacillus cereus* in dairy chilling tanks and optimization of clean-in-place (CIP) regimes using response surface methodology. Food Control 36, 153–158.
Kunst, F., Ogasawara, N., Moszer, I., Albertini, A.M., Alloni, G., Azevedo, V., Bertero, M.G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S.C., Bron, S., Brouillet, S., Bruschi, C.V., Caldwell, B., Capuano, V., Carter, N.M., Choi, S.K., Cordani, J.J., Connerton, I.F., Cummings, N.J., Daniel, R.A., Denziot, F., Devine, K.M., Dusterhoft, A., Ehrlich, S.D., Emmerson, P.T., Entian, K.D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fujita, Y., Fuma, S., Galizzi, A., Galleron, N., Ghim, S.Y., Glaser, P., Goffeau, A., Golightly, E.J., Grandi, G., Guiseppi, G., Guy, B.J., Haga, K., Haiech, J., Harwood, C.R., Henaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M.F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.M., Levine, A., Liu, H., Masuda, S., Mauel, C., Medigue, C., Medina, N., Mellado, R.P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.H., Parro, V., Pohl, T.M., Portelle, D., Porwollik, S., Prescott, A.M., Presecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., Schroeter, R., Scoffone, F., Sekiguchi, J., Sekowska, A., Seror, S.J., Serror, P., Shin, B.S., Soldo, B., Sorokin, A., Tacconi, E., Takagi, T., Takahashi, H., Takemaru, K., Takeuchi, M., Tamakoshi, A., Tanaka, T., Terpstra, P., Togoni, A., Tosato, V., Uchiyama, S., Vandebol, M., Vannier, F., Vassarotti, A., Viari, A., Wambutt, R., Wedler, H., Weitzenegger, T., Winters, P., Wipat, A., Yamamoto, H., Yamane, K., Yasumoto, K., Yata, K., Yoshida, K., Yoshikawa, H.F., Zumstein, E., Yoshikawa, H., Danchin, A., 1997. The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. Nature 390, 249–256.
Lechner, M., Findeiss, S., Steiner, L., Marz, M., Stadler, P.F., Prohaska, S.J., 2011. Proteinortho: detection of (Co-)orthologs in large-scale analysis. BMC Bioinf. 12.
Lindback, T., Mols, M., Basset, C., Granum, P.E., Kuipers, O.P., Kovacs, A.T., 2012. CodY, a pleiotropic regulator, influences multicellular behaviour and efficient production of virulence factors in *Bacillus cereus*. Environ. Microbiol. 14, 2233–2246.
Lindsay, D., van Holy, A., Brözel, V.S., 2005. Spore formation in *Bacillus subtilis* biofilms. J. Food Prot. 68 (4), 860–865.
Liu, M.J., Bayjanov, J.R., Renckens, B., Nauta, A., Siezen, R.J., 2010. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics 11.
Lopez, D., Vlamakis, H., Losick, R., Kolter, R., 2009. Paracrine signaling in a bacterium. Genes Dev. 23, 1631–1638.
Ludecke, C., Jandt, K.D., Siegismund, D., Kujau, M.J., Zang, E., Rettenmayr, M., Bossert, J., Roth, M., 2014. Reproducible biofilm cultivation of chemostat-grown *Escherichia coli* and investigation of bacterial adhesion on biomaterials using a non-constant-depth film fermenter. PLoS One 9 (1), e84837. https://doi.org/10.1371/journal.pone.0084837.
Madslien, E.H., Ronning, H.T., Lindback, T., Hassel, B., Andersson, M.A., Granum, P.E., 2013. Lichenysin is produced by most *Bacillus licheniformis* strains. J. Appl. Microbiol. 115, 1068–1080.
Marchand, S., De Block, J., De Jonghe, V., Coorevits, A., Heyndrickx, M., Herman, L., 2012. Biofilm formation in milk production and processing environments; influence on milk quality and safety. Compr. Rev. Food Sci. Food Saf. 11, 133–147.
McLoon, A.L., Guttenplan, S.B., Kearns, D.B., Kolter, R., Losick, R., 2011. Tracing the domestication of a biofilm-forming bacterium. J. Bacteriol. 193, 2027–2034.
Morikawa, M., Kagihiro, S., Haruki, M., Takano, K., Branda, S., Kolter, R., Kanaya, S., 2006. Biofilm formation by a *Bacillus subtilis* strain that produces gamma-polyglutamate. Microbiology-Sgm 152, 2801–2807.
Okshevsky, M., Tolker-Nielsen, T., Greve, M., Elena, L., Lamela, O., Nilsson, M., Meyer, R.L., 2018. A transposon mutant library of *Bacillus cereus* ATCC 10987 reveals novel genes required for biofilm formation and implicates motility as an important factor for pellicle- biofilm formation. Microbiologyopen 7, 1–17.
Ostrov, I., Harel, A., Bernstein, S., Steinberg, D., Shemesh, M., 2016. Development of a method to determine the effectiveness of cleaning agents in removal of biofilm derived spores in milking system. Front. Microbiol. 7, 1498.
Ostrov, I., Sela, N., Freed, M., Khateb, N., Kott-Gutkowski, M., Inbar, D., Shemesh, M., 2015. Draft genome sequence of *Bacillus licheniformis* S127, isolated from a sheep udder clinical infection. Genome Announc. 3.
Paradis, E., Claude, J., Strimmer, K., 2004. APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20 (2), 289–290.
Parkinson, T.J., Merrall, M., Fenwick, S.G., 1999. A case of bovine mastitis caused by *Bacillus cereus*. N. Z. Vet. J. 47, 151–152.
Parry, J.M., Turnbull, P.C.B., Gibson, J.R., 1983. A Color Atlas of *Bacillus* Species. Wolfe Medical Publications, London.
Pasvolsky, R., Zakin, V., Ostrova, I., Shemesh, M., 2014. Butyric acid released during milk

lipolysis triggers biofilm formation of *Bacillus* species. Int. J. Food Microbiol. 181, 19–27.
Pedrido, M.E., de Ona, P., Ramirez, W., Lenini, C., Goni, A., Grau, R., 2013. Spo0A links de novo fatty acid synthesis to sporulation and biofilm development in *Bacillus subtilis*. Mol. Microbiol. 87, 348–367.
Pratten, J., 2007. Growing oral biofilms in a constant depth film fermentor (CDFF). Curr Protoc Microbiol Chapter 1 Unit 1B 5.
Salvetti, S., Faegri, K., Ghelardi, E., Kolsto, A.B., Senesi, S., 2011. Global gene expression profile for swarming *Bacillus cereus* bacteria. Appl. Environ. Microbiol. 77, 5149–5156.
Sanchez-Vizuete, P., Le Coq, D., Bridier, A., Herry, J.M., Aymerich, S., Briandet, R., 2015. Identification of *ypqP* as a new *Bacillus subtilis* biofilm determinant that mediates the protection of *Staphylococcus aureus* against antimicrobial agents in mixed-species communities. Appl. Environ. Microbiol. 81, 109–118.
Shaheen, R., Svensson, B., Andersson, M.A., Christiansson, A., Salkinoja-Salonen, M., 2010. Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. Food Microbiol. 27, 347–355.
Sharma, M., Anand, S.K., 2002. Characterization of constitutive microflora of biofilms in dairy processing lines. Food Microbiol. 19, 627–636.
Shemesh, M., Chai, Y.R., 2013. A combination of glycerol and manganese promotes biofilm formation in *Bacillus subtilis* via histidine kinase KinD signaling. J. Bacteriol. 195, 2747–2754.
Shemesh, M., Pasvolsky, R., Zakin, V., 2014. External pH is a cue for the behavioral switch that determines surface motility and biofilm formation of *Alicyclobacillus acidoterrestris*. J. Food Prot. 77, 1418–1423.
Simoes, M., Simoes, L.C., Vieira, M.J., 2010. A review of current and emergent biofilm control strategies. Lwt-Food Science and Technology 43, 573–583.
Somers, E.B., Johnson, M.E., Wong, A.C.L., 2001. Biofilm formation and contamination of cheese by nonstarter lactic acid bacteria in the dairy environment. J. Dairy Sci. 84, 1926–1936. https://doi.org/10.3168/jds.S0022-0302(01)74634-6.
Stanley, N.R., Lazazzera, B.A., 2005. Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly- gamma - DL - glutamic acid production and biofilm formation. Mol. Microbiol. 57, 1143–1158. https://doi.org/10.1111/j.1365-2958.2005.04746.x.
Switt, A.I.M., Andrus, A.D., Ranieri, M.L., Orsi, R.H., Ivy, R., den Bakker, H.C., Martin, N.H., Wiedmann, M., Boor, K.J., 2014. Genomic comparison of spore-forming bacilli isolated from milk. BMC Genomics 15.
Teh, K.H., Flint, S., Palmer, J., Andrewes, P., Bremer, P., Lindsay, D., 2012. Proteolysis produced within biofilms of bacterial isolates from raw milk tankers. Int. J. Food Microbiol. 157, 28–34.
Teh, K.H., Lindsay, D., Palmer, J., Andrewes, P., Bremer, P., Flint, S., 2013. Lipolysis within single culture and co-culture biofilms of dairy origin. Int. J. Food Microbiol. 163, 129–135.
Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K.H., Ehrenreich, P., Baumer, S., Henne, A., Liesegang, H., Merkl, R., Ehrenreich, A., Gottschalk, G., 2004. The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J. Mol. Microbiol. Biotechnol. 7, 204–211.
Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., Michiels, J., 2008. Living on a surface: swarming and biofilm formation. Trends Microbiol. 16, 496–506.
Vlamakis, H., Chai, Y.R., Beauregard, P., Losick, R., Kolter, R., 2013. Sticking together: building a biofilm the *Bacillus subtilis* way. Nat. Rev. Microbiol. 11, 157–168.
Wijman, J.G., deLeeuw, P.P., Moezelaar, R., Zwietering, M.H., Abee, T., 2007. Air-liquid interface biofilms of *Bacillus cereus*: formation, sporulation, and dispersion. Appl. Environ. Microbiol. 73, 1481–1488.
Wu, M.C., Chen, Y.C., Lin, T.L., Hsieh, P.F., Wang, J.T., 2012. Cellobiose-specific phosphotransferase system of *Klebsiella pneumoniae* and its importance in biofilm formation and virulence. Infect. Immun. 80, 2464–2472.
Yan, F., Yu, Y., Gozzi, K., Chen, Y., Guo, J.H., Chai, Y., 2017. Genome-wide investigation of biofilm formation in *Bacillus cereus*. Appl. Environ. Microbiol. 83.
Ymele-Leki, P., Houot, L., Watnick, P.I., 2013. Mannitol and the mannitol-specific enzyme IIB subunit activate *Vibrio cholerae* biofilm formation. Appl. Environ. Microbiol. 79, 4675–4683.
Yoo, J., Hardin, M.T., Chen, X.D., 2006. The influence of milk composition on the growth of *Bacillus stearothermophilus*. J. Food Eng. 77, 96–102.
Yu, Y.Y., Yan, F., Chen, Y., Jin, C., Guo, J.H., Chai, Y.R., 2016. Poly-gamma-glutamic acids contribute to biofilm formation and plant root colonization in selected environmental isolates of *Bacillus subtilis*. Front. Microbiol. 7.
Zain, S.N.M., Bennett, R., Flint, S., 2017. The potential source of *B. licheniformis* contamination during whey protein concentrate 80 manufacture. J. Food Sci. 82, 751–756.
Zain, S.N.M., Flint, S.H., Bennett, R., Tay, H.S., 2016. Characterization and biofilm screening of the predominant bacteria isolated from whey protein concentrate 80. Dairy Sci. Technol. 96, 285–295.
Zeigler, D.R., 2011. The genome sequence of *Bacillus subtilis subsp. spizizenii* W23: insights into speciation within the *B. subtilis* complex and into the history of *B. subtilis* genetics. Microbiology-Sgm 157, 2033–2041.
Zeigler, D.R., Pragai, Z., Rodriguez, S., Chevreux, B., Muffler, A., Albert, T., Bai, R., Wyss, M., Perkins, J.B., 2008. The origins of 168, W23, and other *Bacillus subtilis* legacy strains. J. Bacteriol. 190, 6983–6995.