2. הדו"ח המפורט

<u>א. דף פותח לדו"ח</u>

<u>שם התוכנית:</u> פיתוח ויישום טכנולוגיה אנטימיקרוביאלית לעיכוב התבססות חיידקים במזון.

Developing and application of novel antimicrobial technology mitigating bacterial establishment in food

<u>קוד הזיהוי:</u> 421-0306-18

<u>קוד מדען:</u> 20-14-0027

השנה הקלנדרית עליה מוגש הדו"ח: 2018

שנת הדו"ח שלישית מתוך סה"כ 3 שנים.

<u>שמות החוקרים בקבוצות המחקר:</u>

ד"ר משה שמש, המחלקה לאיכות מזון ובטיחותו, מינהל המחקר החקלאי, מרכז וולקני.
moshesh@volcani.agri.gov.il

פרופ' בעז פוקרוי, המחלקה להנדסה ולמדע של חומרים, הטכניון מכון טכנולוגי לישראל.
bpokroy@tx.technion.ac.il

<u>התקציר:</u>

1 . הצגת הבעיה

היווצרות קהילות רב תאיות של חיידקים המכונות ביופילמים במערכות של תעשיית המזון מקנה לחיידקים עמידות מוגברת בפני טיפולים אנטימיקרוביאליים ולכן מהווה בעיה קשה בתחום של איכות מזון ובטיחותו. חיידקי הביופילם עלולים לגרום לנזקים ופגיעות משמעותיות הן לאיכות המזון והן לציוד המשמש לייצור המזון. לכן, קיים צורך מידי לפיתוח טכנולוגיות להפחתת היווצרות ביופילמים הנוצרים במזון ובציוד.

2 . מטרות המחקר

מטרת המחקר היא פיתוח טכנולוגיה חדשנית למניעת או/ו הפחתת התבססות חיידקים במזון תוך פגיעה במנגנוני הצמדות או/ו הישרדות של מיקרואורגניזמים המזהמים את המזון.

3 . שיטות העבודה

ציפוי משטחים נעשה באמצעות נידוף תרמי של פחמימנים בעלי תכונות סופר-הידרופוביות. המשטחים אופיינו ע"י מיקרוסקופיה קונפוקלית ,אלקטרונית ודיפרקציית קרני איקס. איכות דוגמאות החלב שהודגרו עם המשטחים נבחנה ע"י בדיקת אחוז חלבון לפי שיטת קיילדל, אחוז שומן לפי שיטת גרבר והתגבנות החלב בעזרת מכשיר אופטיגרף. היעילות למניעת היווצרות הביופילם אובחנה ע"י אפיון מורפולוגי באמצעות צביעה פלואורסנטית ואנליזת מיקרוסקופיה קונפוקלית.

4 . תוצאות עיקריות

פותחו ציפויי משטח אנטימיקרוביאליים באמצעות נידוף תרמי של שעוות פרפין כגון $C_{24}F_{50}$ ו- $C_{36}H_{74}+C_{50}H_{102}$ אשר הראו יציבות סבירה לאחר חשיפתם לדוגמאות החלב. למשטחים אנטימיקרוביאליים שפותחו אין השפעה על איכות טכנולוגית של החלב, כגון אחוז חלבון ושומן או זמן התגבנות וחוזק הגבן. כמוכן, המשטחים הינם בעלי יכולת למנוע היווצרות הביופילם על ידי מספר תבדידים של חיידקי הבצילוס וחיידקי הפסאודומונס.

5 . מסקנות והמלצות לגבי יישום התוצאות.

הצלחנו לפתח דור שני של משטחים אנטימיקרוביאליים הרלוונטיים לתעשיית המזון. משטחים המצופים ב- $C_{24}F_{50}$-ו $C_{36}H_{74}+C_{50}H_{102}$ מראים תוצאות מבטיחות ביציבות במגע עם החלב ומבחינת חוסר השפעה על איכות טכנולוגית של חלב. כמוכן, משטחים אלו מתאפיינים ביכולת במניעת יצירת הביופילם ע"י חיידקים הרוולנטים לתעשיית החלב.

מעריכים מומלצים לבדיקת הדוח המדעי

1. פרופ' יחזקאל קשי (הטכניון)
2. ד"ר ויקטור רודוב (מינהל המחקר החקלאי)
3. פרופ' מיטל רכס (האוניברסיטה העברית)

תוכן העניינים

עמוד

דף פותח לדו"ח..1
שמות החוקרים בקבוצת מחקר...1
התקציר..1
גוף דו"ח מחקר...4
מבוא..4
מטרות המחקר..6
פירוט עיקרי הניסויים שבוצעו וכלל התוצאות שהתקבלו לתקופת הדו"ח 6
דיון...13
ביבליוגרפיה...16

ב. גוף דו"ח המחקר

Introduction

Despite advances in food preservation techniques, bacterial spoilage remains a leading cause of global food. Nearly one-third of all food produced worldwide is estimated to be lost postharvest, and much of this loss can be attributed to microbial spoilage (Ranieri et al., 2012). Dairy products constitute one of the leading sectors impacted by food loss, as nearly 20% of conventionally pasteurized fluid milk is discarded prior to consumption and overall estimated yearly loss of over 30 billion dollars in US and 330 million dollars in Israel (Buzby et al., 2014; Chen Herzog et al., 2019; Ivy et al., 2012; Ranieri et al., 2012). It appears that the major source of the contamination of dairy products is often associated with biofilms formed on the surfaces of milk processing equipment (Flint et al., 1997). Biofilms are highly structured multicellular communities, which allow bacteria to survive in hostile environments (Hall-Stoodley et al., 2004; Kolter and Greenberg, 2006). Biofilms are not only a potential source of contamination, but can also increase corrosion rate of metal pipes and equipment used in the milk industry, reduce heat transfer and increase fluid frictional resistance (Kumar and Anand, 1998). Thus, contamination of dairy products due to the presence of bacterial biofilms is a major concern to modern dairy manufacturers, especially with current trends towards longer production runs, the use of complex equipment, the automation of plants and increasingly stringent microbiological requirements.

Bovine milk is highly nutritious, which makes it an ideal medium for the growth of microorganisms. It contains abundant water and nutrients (such as lactose, proteins, and lipids) and has a nearly neutral pH. Since microorganisms in milk may hold spoilage and/or health risks, milk manufacturing is subject to extremely stringent regulations. These regulations include pasteurization at high temperatures, which kills most bacteria, and milk storage at low temperatures, which limits the growth of many bacteria. In addition, dairy farm pipelines are regularly cleaned with alkaline and acidic liquids at high temperatures in a cleaning-in-place (CIP) procedure (Bremer et al., 2006; Ostrov et al., 2016). Despite these stringent conditions, persistent bacteria are able to overcome these treatments. For instance, thermophilic and spore-

forming bacteria are able to survive and thrive milk processing and storage (Ostrov et al., 2019a; Simoes et al., 2010). Moreover, bacterial spores can survive treatment with reagents commonly used in CIP procedures (Shaheen et al., 2010). Some of these bacteria produce enzymes (proteases and lipases), resulting in off-flavors and curdling in the final product (Ranieri et al., 2012; Teh et al., 2013).

Members of the *Bacillus* genus are of the most common bacteria found in dairy farms as well as processing plants (Sharma and Anand, 2002; Simoes et al., 2010), and are the predominant Gram-positive bacteria isolated from raw and pasteurized milk (Meer et al., 1991; Ranieri et al., 2009). They form abundant biofilms on stainless steel, commonly used in food processing plants, and contribute to biofouling of processed food (Ostrov et al., 2019b; Peng et al., 2002). As *Bacillus* species are ubiquitously present in nature, they easily spread through food production systems, and contamination with these species is almost inevitable. Moreover, *Bacillus* spores are both highly resistant to a variety of stresses and very hydrophobic, which allows them to adhere easily to food processing equipment (Lindsay et al., 2002). In *B. subtilis*, biofilm and spore formation are closely linked as mature spores can constitute up to 20% of the total cells in a biofilm (Branda et al., 2001; Chai et al., 2010). Biofilms formed by thermo-resistant *Bacillus* species in a milk line can rapidly grow to such an extent that the passing milk is contaminated with cells released from the biofilm (Wirtanen et al., 1996). Thus, biofilms formed by *Bacillus* species are a major type of hygiene problems in dairy industry.

The main strategy currently applied to prevent biofilm formation in this industry is to regularly clean and disinfect work surfaces before bacteria become firmly attached to them (Ostrov et al., 2016). Once microbial cells have become attached to surfaces through a biofilm, their removal can be extremely challenging (Ostrov et al., 2016). Clearly, preventing the formation of biofilms would be a much more desirable option than eliminating them as they mature. To date, however, there is no technique known to successfully prevent or control biofilm formation without causing adverse side effects. We therefore thrilled to develop an effective strategy to prevent dairy biofilm formation that

involve passively inhibiting adhesion of the microorganisms to work surfaces of dairy equipment.

Research goals

The main objective of this project was to develop an effective strategy to prevent dairy biofilm formation that would mitigate adhesion of the microorganisms to work surfaces of dairy equipment. Thus, we aimed to develop the antifouling superhydrophobic surfaces, formed via the self-assembly of paraffin or fluorinated wax crystals, which prevent biofilm formation on different substrates. We further tested those surfaces under conditions relevant to dairy food production, for instance their ability to mitigate biofilm development by dairy *Bacillus* isolates, which are among the most detrimental formers of dairy biofilms. In addition, we aimed to confirm that the developed wax-coated surfaces had no negative effects on the technological quality of the milk.

Detailed description of the experiments and results of the research

1. Newly generated $C_{24}F_{50}$-coated and $C_{36}H_{74}$+$C_{50}H_{102}$-coated surfaces are characterized by higher surface roughness and denser distribution of wax crystals.

We chose the $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ hierarchical wax surfaces for this study because these structures were previously shown to significantly resist attachment and biofilm formation by different pathogenic bacteria (Pechook et al., 2015). In the current study we tested those wax-coated surfaces against milk-derived *Bacillus* isolates (Ostrov et al., 2019b). We chose to use these bacteria to evaluate the developed surfaces because of their highly robust biofilm formation (Ostrov et al., 2019b) and their relevance to dairy industry.

During the course of our present experiments we found, however, that the coatings fabricated according to the previously reported procedure (Pechook et al., 2015) could not fully withstand the robust biofilm formation by the tested bacteria (Figure S1). We therefore attempted to optimize the quality of the $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ coatings by modifying the conditions of the wax evaporation process. Accordingly, we doubled the weight of the wax that we had used under the initial evaporation conditions.

We performed complex characterization of the $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ wax surfaces that were generated under the initial and the optimized evaporation conditions (Figure 1, Figure S2).

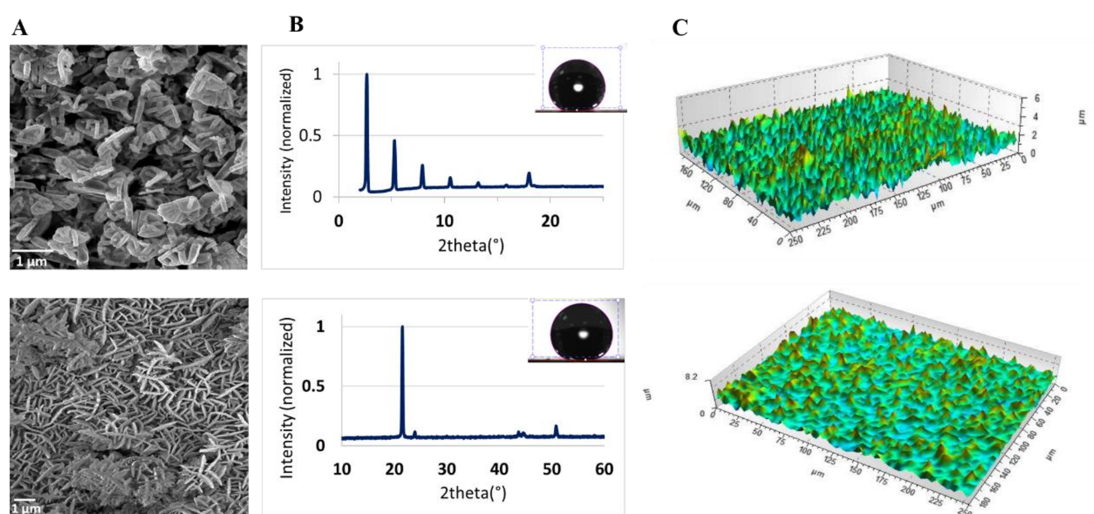

Figure 1. Complex characterization of elaborated $C_{24}F_{50}$ (top panel) and $C_{36}H_{74}$+$C_{50}H_{102}$ (bottom panel) wax surfaces. (A) SEM images of wax crystals structure and distribution; (B) XRD pattern collected from the tested surface; inset – contact angle between water droplet and the tested wax surface; (C) confocal microscopy 3D topographic images.

The results confirmed the presence of thermally deposited $C_{24}F_{50}$ or $C_{36}H_{74}$+$C_{50}H_{102}$ wax films on the surfaces of the substrates (steel) under both sets of evaporation conditions (Figure 1, Figure S2). These wax films conferred superhydrophobic properties on the surfaces, with mean contact angles of 168 °C for $C_{24}F_{50}$ and 171 °C for $C_{36}H_{74}$+$C_{50}H_{102}$ (Figure 1B, inset; Figure S2C). However, the wax coatings that were generated under optimized evaporation conditions demonstrated significantly higher surface roughness (0.6 and 0.8 microns compared to 0.147 and 0.232 microns for $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$, respectively; Figure 1C; Figure S2D). XRD patterns collected from the surfaces demonstrated that the layers exhibit a preferred orientation, as shown by the relatively prominent peak at 2θ~2.5 ° for $C_{24}F_{50}$ and 2θ~21 ° for $C_{36}H_{74}$+$C_{50}H_{102}$ (attributed to the (001) planes; Figure 1B, Figure S2B). SEM images of both $C_{24}F_{50}$-coated and $C_{36}H_{74}$+$C_{50}H_{102}$-coated surfaces revealed hierarchical structures composed of wax crystals of different sizes (Figure 1A, Figure S2A). Notably, we observed a significantly denser distribution of highly oriented wax

crystals over the surfaces that were generated using the optimized evaporation conditions (Figure 1A, Figure S2A). As a consequence, the $C_{24}F_{50}$-coated and $C_{36}H_{74}$+$C_{50}H_{102}$-coated surfaces obtained under the optimized evaporation conditions demonstrated higher surface roughness and enhanced hierarchies, which also resulted in their improved resistance to mechanical damage.

2. $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ coated wax surfaces strongly mitigate biofilm formation by the dairy-associated *Bacillus*. We further examined the optimized wax surfaces for their ability to prevent biofilm formation by the dairy-associated bacterial strain *B. paralicheniformis* S127, which is normally characterized by extremely robust biofilm formation (Ostrov et al., 2019b; Ostrov et al., 2015). This analysis was performed under previously described conditions (LB medium, batch culture) (Pechook et al., 2015). CSLM images showed that *B. paralicheniformis* S127 was characterized by robust biofilm development on uncoated (control) steel, glass and polystyrene substrates during both short-term (48 h) and long-term (7 d) cultivation, whereas the $C_{24}F_{50}$-coated and $C_{36}H_{74}$+$C_{50}H_{102}$-coated surfaces drastically inhibited bacterial attachment and biofilm formation throughout all tested conditions (Figures 2A, 3A; Tables S1, S2).

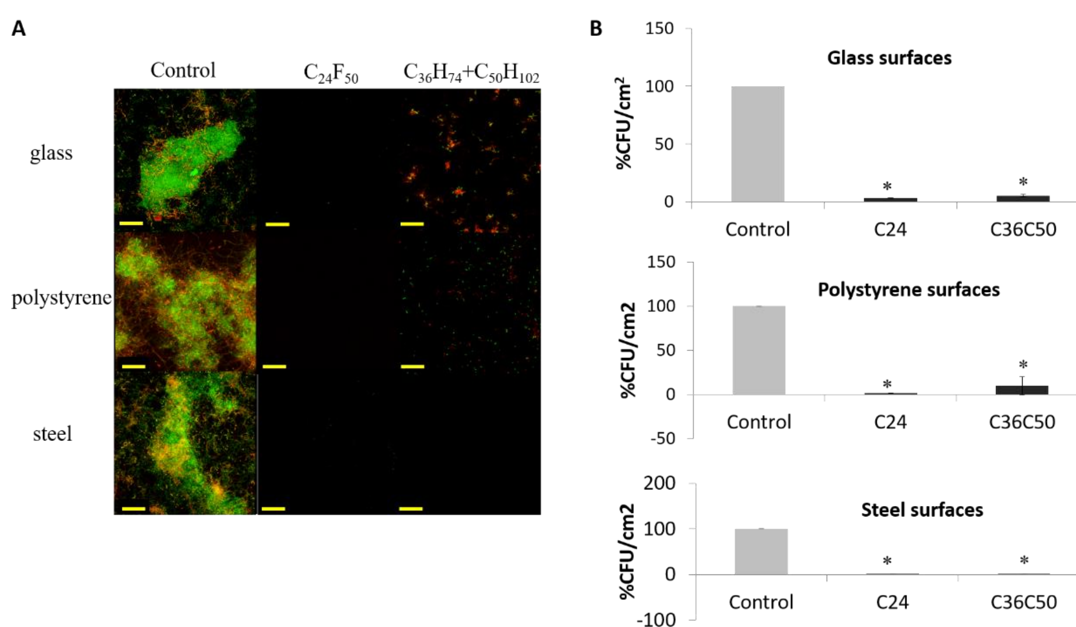

Figure 2. (A) CLSM images of *B. paralicheniformis* S127 biofilms formed on $C_{24}F_{50}$, and $C_{36}H_{74}$+$C_{50}H_{102}$ polymer surfaces, thermally evaporated on stainless steel, glass and polystyrene substrates, after 48 h of cultivation. Biofilms generated on glass, stainless steel and polystyrene substrates in LB medium at 30 °C, 25 rpm for 48 h,

were stained using the FilmTracerTM LIVE/DEAD Biofilm Viability Kit. Live cells stained green, dead cells stained red. Stained samples were visualized by CSLM at 50 µm scale. The images represent typical results of two different biological experimental repeats performed in triplicate. (B) Live bacterial counts of *B. paralicheniformis* S127 on uncoated steel, glass or polystyrene surfaces (control) and on surfaces coated with $C_{24}F_{50}$ or $C_{36}H_{74}$+$C_{50}H_{102}$ after 48 h of cultivation. The results are means and SD of two different biological experimental repeats performed in triplicate. * – statistically significant difference ($P < 0.05$) between viable cell counts in a given sample versus viable cell counts in non-coated sample (control).

To support the results obtained by CLSM analysis, we used the plating method to quantify colony-forming units (CFU) of viable bacteria adhering to the $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ wax surfaces formed on glass, stainless steel, and polystyrene substrates. Compared to control surfaces, reductions of 90% to 99% were observed in the quantities of *B. paralicheniformis* cells adhering to $C_{24}F_{50}$-coated or $C_{36}H_{74}$+$C_{50}H_{102}$-coated substrates after both 48 h and 7 d of incubation (Figure 2B, Figure 3B).

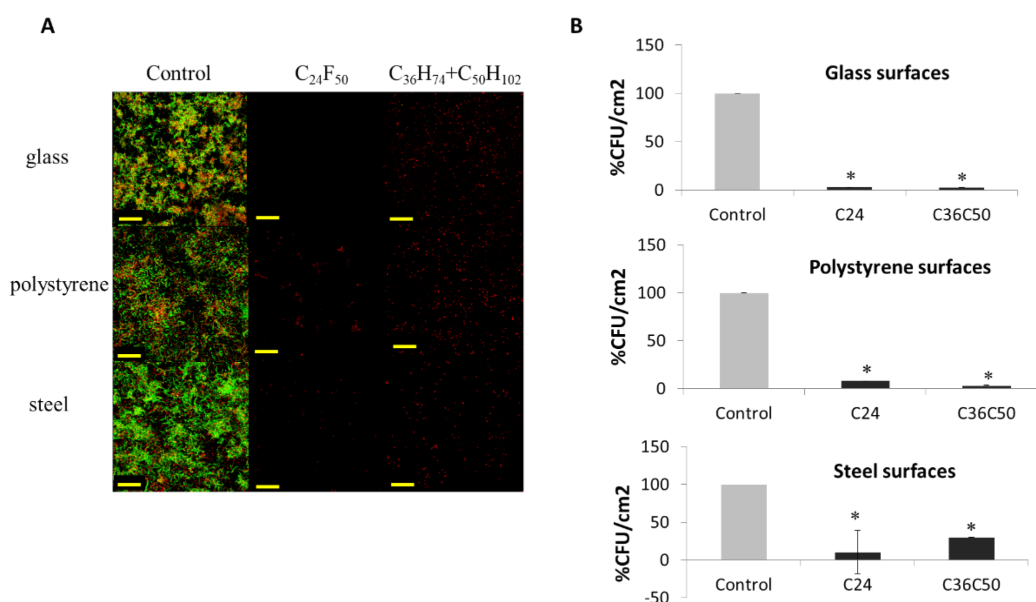

Figure 3. (A) CLSM images of *B. paralicheniformis* S127 biofilms formed on $C_{24}F_{50}$, and $C_{36}H_{74}$+$C_{50}H_{102}$ polymer surfaces, thermally evaporated on stainless steel, glass and polystyrene substrates, after 7d of cultivation. Biofilms generated on glass, stainless steel and polystyrene substrates in LB medium at 30 °C, 25 rpm for 7d, were stained using the FilmTracerTM LIVE/DEAD Biofilm Viability Kit. Live cells stained green, dead cells stained red. Stained samples were visualized by CSLM at 50 µm scale. The images represent typical results of two different biological experimental repeats performed in triplicate. (B) Live bacterial counts of *B. paralicheniformis* S127 on uncoated steel, glass or polystyrene surfaces (control) and on surfaces coated with

$C_{24}F_{50}$ or $C_{36}H_{74}$+$C_{50}H_{102}$ after 7d of cultivation. The results are means and SD of two different biological experimental repeats performed in triplicate. * – statistically significant difference ($P < 0.05$) between viable cell counts in a given sample versus viable cell counts in non-coated sample (control).

Another important aim of our study was to find out whether surface modification with wax coatings also affects biofilm formation in other strong biofilm-forming dairy *Bacillus* isolates (isolated from Israeli dairy farms situated in different regions of Israel) (Ostrov et al., 2019b). CLSM analyses revealed significant inhibition of biofilm formation on $C_{24}F_{50}$-coated and $C_{36}H_{74}$+$C_{50}H_{102}$-coated steel substrates by all of the additional *Bacillus* strains tested (Figure S3, Table S3). This finding pointed to the potential ability of the $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ hierarchical structures to resist surface attachment and the subsequent biofilm development independently of any specific chemical or physical feature of any of the tested bacterial cells.

Altogether, our results indicated that $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ wax surfaces provide effective inhibition of bacterial adhesion and biofilm formation by dairy-associated *Bacillus* strains on different types of substrates.

3. The generated surfaces effectively mitigate biofilm formation in milk. To evaluate the potential applicability of the $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ wax surfaces in the dairy industry, we examined their ability to resist biofilm formation by the dairy-associated *Bacillus* isolates within milk. We used a constant depth film fermentor (CDFF), the growth system to simulate conditions of the actual dairy environment, which are more conducive to strong biofilm formation (Ostrov et al., 2019b).

We investigated the effects of the tested wax surfaces on biofilms formed in milk by *B. paralicheniformis* S127, *B. licheniformis* MS310, *B. paralicheniformis* MS303, *B. subtilis* MS302 and *B. licheniformis* MS307. Following incubation in the CDFF, all of the tested strains formed extremely robust biofilms on uncoated PTFE plugs (control), while $C_{24}F_{50}$- and $C_{36}H_{74}$+$C_{50}H_{102}$-coated plugs demonstrated strong inhibition of biofilm formation (Figure 4A, Table S4). Likewise, the results of CFU quantitation indicated a reduction of 96% to 99% in the numbers of bacteria adhering to $C_{24}F_{50}$- or $C_{36}H_{74}$+$C_{50}H_{102}$-coated plugs relative to the control (Figure 4B).

These results thus showed that $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ wax coatings effectively prevent biofilm formation by dairy-associated *Bacillus* strains in milk under the harsh culture conditions resembling a dairy environment (continuous flow of milk in the system).

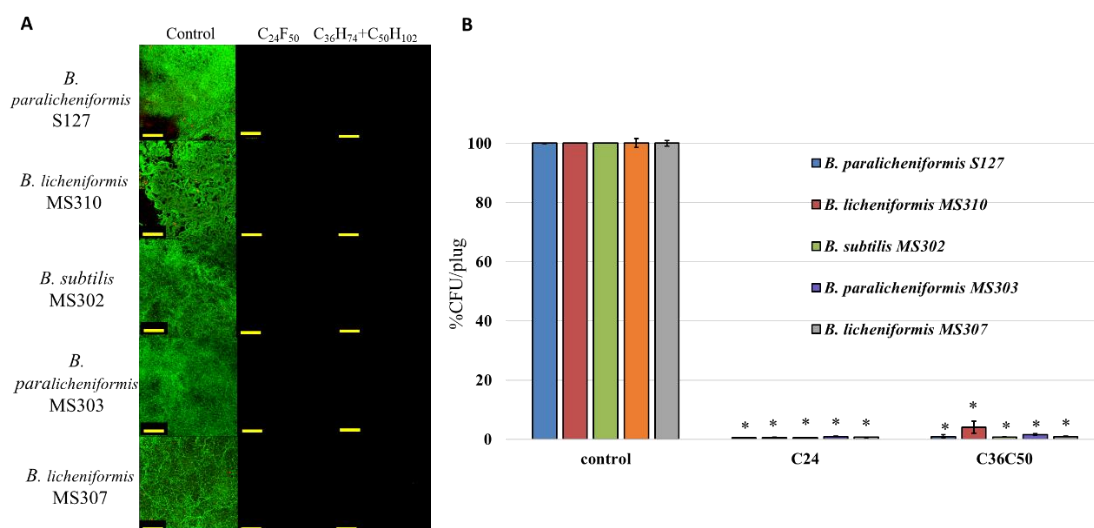

Figure 4. Biofilm formation on $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ polymer surfaces in milk following cultivation in the CDFF (A) CLSM images of *B. paralicheniformis* S127, *B. licheniformis* MS310, *B. paralicheniformis* MS303, *B. subtilis* MS302 and *B. licheniformis* MS307 biofilms generated on $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ polymer surfaces, thermally evaporated on PTFE substrates. The images represent typical results of two different biological experimental repeats performed in triplicate. Scale – 50 µm. (B) Live bacterial counts of *B. paralicheniformis* S127, *B. licheniformis* MS310, *B. paralicheniformis* MS303, *B. subtilis* MS302 and *B. licheniformis* MS307 on control surfaces and on surfaces coated with $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ after 24h of cultivation in the CDFF. The results are means and SD of two different biological experimental repeats performed in triplicate. * – statistically significant difference ($P < 0.05$) between viable cell counts in a given sample versus viable cell counts in non-coated sample (control).

4. The generated surfaces maintain their superhydrophobic properties during exposure to milk. To further test the potential applicability of the $C_{24}F_{50}$- and $C_{36}H_{74}$+$C_{50}H_{102}$-coated surfaces in the dairy industry, we evaluated their stability during contact with milk. According to our data, exposure of the surfaces to raw milk led to an increase in their surface roughness (1.74 microns for $C_{24}F_{50}$ and 0.95 microns for $C_{36}H_{74}$+$C_{50}H_{102}$; Figure 5C) and changes in the

preferred orientation of wax crystals deposited on those surfaces (Figure 5B). However, both the $C_{24}F_{50}$- and the $C_{36}H_{74}$+$C_{50}H_{102}$-coated surfaces remained superhydrophobic, with mean water-contact angles of about 150 °C (Figure 5B inset). Furthermore, SEM images revealed dense distribution of wax crystals on the examined surfaces with no areas of damage after the incubation tests (Figure 5A). Thus, these results proved that and $C_{36}H_{74}$+$C_{50}H_{102}$ wax coatings remain stable despite their long-term exposure to milk.

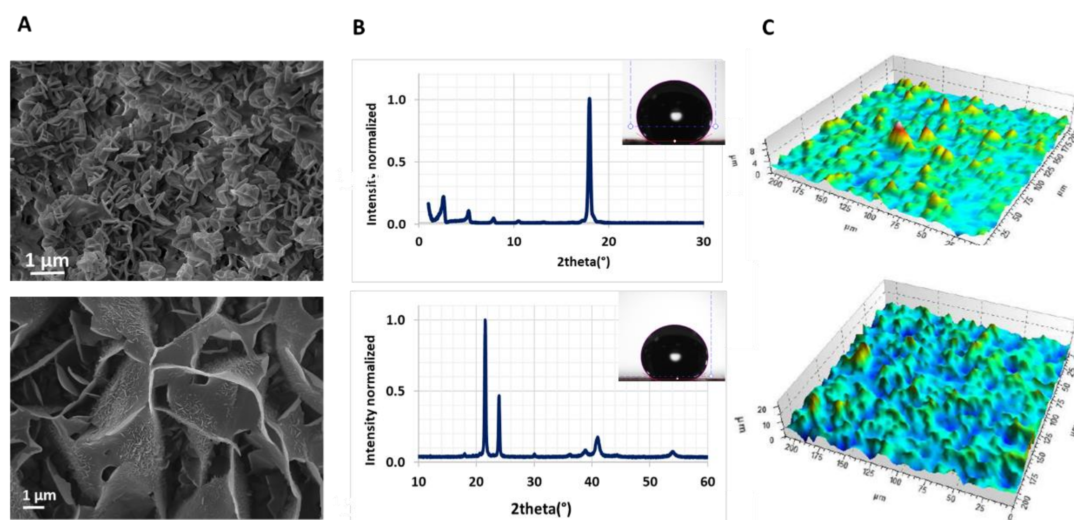

Figure 5. Complex characterization of $C_{24}F_{50}$ (top panel) and $C_{36}H_{74}$+$C_{50}H_{102}$ (bottom panel) surfaces after 4 days of incubation in milk. (A) SEM images of wax crystals structure and distribution; (B) XRD pattern collected from the tested surface, inset – contact angle between water droplet and the tested wax surface; (C) confocal microscopy 3D topographic images.

5. The generated surfaces have no effect on the technological quality of milk. An important aspect of our study was to evaluate the technological quality of milk (such as protein, fat and dry matter content, and curdling) exposed to the wax surfaces. According to our results, exposure to $C_{24}F_{50}$- or $C_{36}H_{74}$+$C_{50}H_{102}$-coated surfaces for either 1 or 4 days did not affect protein, fat or dry matter content (Figure 6A, D, E) in the milk. Moreover, incubation with the tested surfaces had no effect on milk curdling parameters, such as start of curdling or curdle strength (Figure 6B, C).

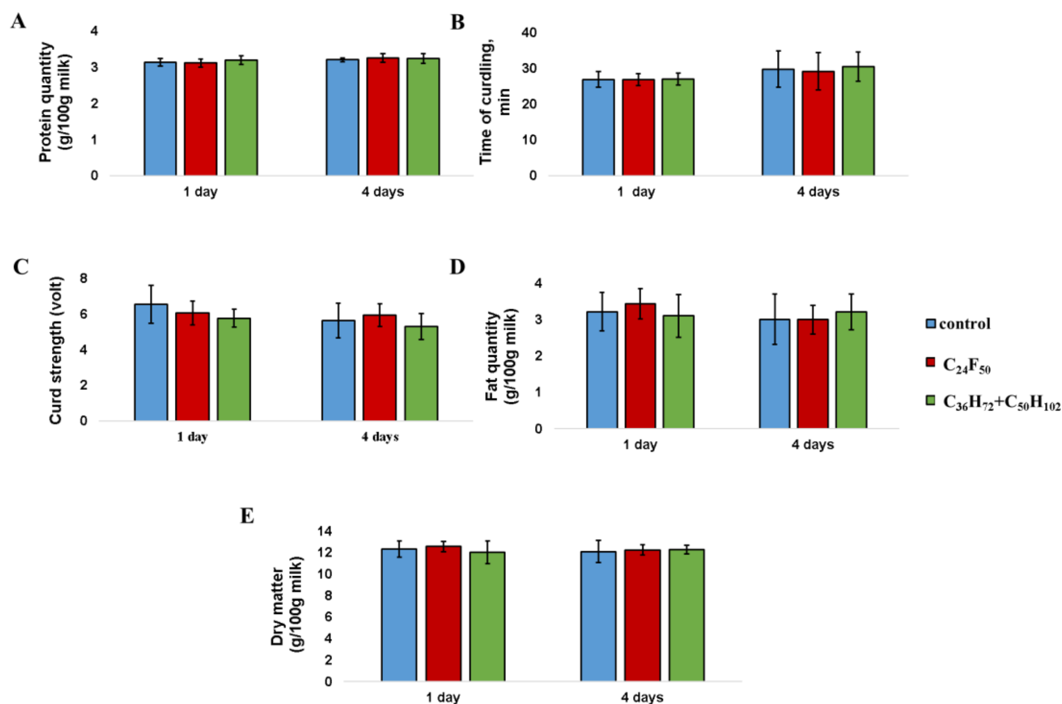

Figure 6. Effect of $C_{24}F_{50}$ and $C_{36}H_{74}+C_{50}H_{102}$ surfaces on technological properties of milk products after 1 and 4 days of incubation. (A) – protein content; (B) – time of curdling; (C) – curd strength; (D) – fat content; (E) – dry matter content. This experiment was performed similarly as reported previously (Ben-Ishay et al., 2017). The results are means and SD of two different biological experimental repeats performed in duplicate.

Discussion

Findings of this study provide evidence of developing an accessible anti-biofilm technology based on the successful formation of superhydrophobic paraffin or fluorinated wax coatings on different kinds of substrates. The quality of the $C_{24}F_{50}$ and the $C_{36}H_{74}+C_{50}H_{102}$ coatings was successfully improved by doubling the weight of the wax applied on the substrates, thereby increasing both the density of wax crystal distribution and the roughness of substrate surfaces. Importantly, doubling the weight of the wax facilitated creation of a hierarchical surface structure. According to previous studies, an increase in surface hierarchy leads to higher stability of the Cassie wetting state, which might be beneficial for long-term antifouling capabilities (Nosonovsky and Bhushan, 2007; Pechook et al., 2015). In the case of $C_{36}H_{74}+C_{50}H_{102}$, moreover, optimized evaporation conditions facilitated superposition of several roughness

patterns over different length scales, resulting in the formation of a double roughness profile capable of further enhancing hydrophobicity (Sun et al., 2005). Finally, optimized evaporation conditions resulted in higher resistance to mechanical damage; such resistance is a fundamental requirement for ensuring sustained protection against bacterial adhesion. All in all, we showed that the tested wax surfaces, when generated under optimized evaporation conditions, efficiently prevent both short-term and long-term biofilm formation by several aggressive biofilm-forming dairy *Bacillus* strains (Figures 2, 3).

We further demonstrated that $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ wax surfaces effectively mitigate biofilm formation in milk (Figure 4). To the best of our knowledge, inhibition of biofilm formation in milk by antimicrobial/anti-biofilm surfaces has not been previously reported. It should be noted that biofilm cultivation in milk in the present study took place in a continuous flow system (CDFF; Figure 4), in which conditions are more hostile than those in a batch culture set-up (owing to exposure of the coatings to tangential abrasive forces and the need to withstand dynamic impact (Sebastian et al., 2018), and closer to actual conditions resembling a industrial environment (continuous flow of milk in the system) (Ostrov et al., 2019a).

The effect of $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ on biofilm formation can probably be explained by their impairment of bacterial surface attachment, with resulting superhydrophobicity and more favorable surface topography (Damodaran and Murthy, 2016). It has indeed been shown that hydrophobicity correlates with flow-induced detachment of bacteria (Salta et al., 2010), which can be rather beneficial during cleaning procedures in the dairy-associated environment.

A further useful finding of our study was that the wax coatings could retain their structure during bacterial incubation in milk (Figure 5), an aspect that, to our knowledge, also has not been previously tested. In this connection, to ensure sustained protection against bacterial adhesion it is crucial that the superhydrophobic behaviour of the coatings remains intact. Any damage to the engineered structures might result in significant deviation in the water-repellent capability of the coating (Sebastian et al., 2018), thus probably also affecting the anti-biofilm capability. Furthermore, exposure to $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$

wax coatings were found to have no negative influence on milk technological quality, a crucial requirement for their potential application in the dairy industry.

The results obtained here with $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ wax surfaces are comparable to (and may even exceed) recently reported benefits with other state-of-the-art antibacterial surfaces. Thus, epoxy/Ag-TiO2 composites developed by Santhosh and Natarajan demonstrated 67%–77% inhibition of biofilm formation by *Escherichia coli* and *Staphylococcus aureus,* respectively, after 18 h of exposure (Santhosh and Natarajan, 2015). Silver nanoparticles that were combined with leaf extract of *Allophylus cobbe* and conventional antibiotics showed high antibacterial and antibiofilm activities (about 95% reduction in biofilm as indicated by crystal violet staining) against *P. aeruginosa* and *S. aureus (Gurunathan et al., 2014).* Exposure to the tryptophan-rich peptide TetraF2W-RR resulted in 83% reduction in adhering of bacterial cells after 24 h of exposure (Mishra et al., 2016). Finally, on 2,3,5,6-tetrafluoro-p-phenylenedimethanol-coated polyethylene terephthalate sheets developed by Bao et al., attachment of *B. subtilis* and *E. coli* was reduced, after 72 h of incubation, by 27.6% to 89.2% respectively (Bao et al., 2017). The effects of the abovementioned antibacterial surfaces in a dairy environment, however, have never been tested. Furthermore, certain drawbacks could limit the potential use of these surfaces in the dairy industry. Metallic nanoparticles can be toxic to humans when ingested, and their bactericidal effect can lead to the development of resistance in the bacteria (Martirosyan et al., 2014). Titanium-based materials need to be UV-irradiated since their bactericidal activity derives from their photocatalytic effect (Santhosh and Natarajan, 2015). *In vitro*, considerable experimental data testify to mechanisms by which bacteria may develop resistance toward surface-immobilized antimicrobial peptides under selection pressure (Pranting et al., 2008). Furthermore, milk proteins or dead bacteria may block the antimicrobial groups on the surface of a peptide-based coating (Riool et al., 2017). In contrast, $C_{36}H_{74}$+$C_{50}H_{102}$ and $C_{24}F_{50}$ do not require activation before exposure to bacteria, and do not demand concomitant antimicrobial treatment if their activity needs to be boosted. Moreover, as shown in Figure 5, superhydrophobic properties of the tested coatings were retained after exposure of the surfaces to whole milk components. Importantly, some of the thermophilic bacteria can grow and form biofilm at elevated temperatures

such as 50 – 65°C, which fits with the temperatures used during cleaning procedures of milking systems (or dairy parlor) and certain technological processes of dairy products manufacture (Ostrov et al., 2016). To this end, the melting temperature of $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ surfaces are 189°C, 76.5°C and 94°C respectively, which indicates high probability of surfaces remaining intact at the above-mentioned temperature range. However, it would be of interest to test the stability of the coated surfaces at the elevated temperatures.

Importantly, our additional data indicate of no significant influence on $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ coatings stability under turbulent flow in the course of cleaning procedures (as tested in the cleaning-in-place (CIP) model system (Ostrov et al., 2016)) (data not shown). Since the coating procedures of the stainless steel (a major substrate used in the dairy industry) would not be very costly, it could be replaced or renewed relatively often. This might prevent undesired effects of milk fouling, by raw as well as processed milk, on the coated surfaces. Finally, our data indicate that there would not be any significant influence or release of the wax coatings in the milk products. All of these reasons can be assumed to affirm the usage potential of superhydrophobic wax surfaces in the dairy industry. Even though, the point of regulation and safety of using those coatings in food industry would be further investigated in future studies.

References

Bao, Q., Nishimura, N., Kamata, H., Furue, K., Ono, Y., Hosomi, M., and Terada, A., 2017. Antibacterial and anti-biofilm efficacy of fluoropolymer coating by a 2,3,5,6-tetrafluoro-p-phenylenedimethanol structure. *Colloids and Surfaces B-Biointerfaces* 151.363-371:

Ben-Ishay, N., Oknin, H., Steinberg, D., Berkovich, Z., Reifen, R., and Shemesh, M., 2017. Enrichment of milk with magnesium provides healthier and safer dairy products. *npj Biofilms and Microbiomes* 3.

Branda, S.S., Gonzalez-Pastor, J.E., Ben-Yehuda, S., Losick, R., and Kolter, R., 2001. Fruiting body formation by *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* 98:11621-11626.

Bremer, P.J., Fillery, S., and McQuillan, A.J., 2006. Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *Int J Food Microbiol* 106:254-262.

Buzby, J.C., Hodan F. Wells, and Hyman, J., 2014. The Estimated Amount, Value, and Calories of Postharvest Food Losses at the Retail and Consumer Levels in the United States, EIB-121. U.S. Department of Agriculture, Economic Research Service.

Chai, Y., Kolter, R., and Losick, R., 2010. Reversal of an epigenetic switch governing cell chaining in *Bacillus subtilis* by protein instability. *Mol. Microbiol.*78:218-229 .

Chen Herzog, Matan Nahaissi, Azoulay, E., and Lazaresko, N.W., 2019. Food waste and rescue in Israel - The economic, social and environmental impact. Leket Israel and BDO consulting Israel.

Damodaran, V.B. and Murthy, N.S., 2016. Bio-inspired strategies for designing antifouling biomaterials. *Biomater Res* 20:18.

Flint, S.H., Bremer, P.J., and Brooks, J.D., 1997. Biofilms in dairy manufacturing plant - description, current concerns and methods of control. *Biofouling* 11:81-97.

Gurunathan, S ,.Han, J.W., Kwon, D.N., and Kim, J.H., 2014. Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. *Nanoscale Research Letters* 9.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P .Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2:95-108.

Ivy, R.A., Ranieri, M.L., Martin, N.H., den Bakker, H.C., Xavier, B.M., Wiedmann, M., and Boor, K.J., 2012. Identification and characterization of psychrotolerant sporeformers associated with fluid milk production and processing. *Appl Environ Microbiol* 78:1853-1864.

Kolter, R. and Greenberg, E.P., 2006. Microbial sciences: the superficial life of microbes. *Nature* 441:300-302.

Kumar, C.G. and Anand, S.K .1998 ,.Significance of microbial biofilms in food industry: a review. *Int J Food Microbiol* 42:9-27.

Lindsay, D., Brozel, V.S., Mostert, J.F., and von Holy, A., 2002. Differential efficacy of a chlorine dioxide-containing sanitizer against single species and binary biofilms of a dairy-associated *Bacillus cereus* and a *Pseudomonas fluorescens* isolate. *J Appl Microbiol* 92:352-361.

Martirosyan, A., Bazes, A., and Schneider, Y.J., 2014. In vitro toxicity assessment of silver nanoparticles in the presence of phenolic compounds - preventive agents against the harmful effect? *Nanotoxicology* 8:573-582.

Meer, R.R., Baker, J., Bodyfelt, F.W., and Griffiths, M.W., 1991. Psychrotropic *Bacillus* spp. in fluid milk products: a review. . *Journal of Food Protection* 54:969.979-

Mishra, B., Golla, R.M., Lau, K., Lushnikova, T., and Wang, G.S., 2016. Anti-*Staphylococcal* Biofilm Effects of Human Cathelicidin Peptides. *Acs Medicinal Chemistry Letters* 7:117-121.

Nosonovsky, M. and Bhushan, B., 2007. Hierarchical roughness optimization for biomimetic superhydrophobic surfaces. *Ultramicroscopy* 107:969-979.

Ostrov, I., Harel, A., Bernstein, S., Steinberg, D., and 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., Paz, T., and Shemesh, M., 2019a. Robust Biofilm-Forming *Bacillus* Isolates from the Dairy Environment Demonstrate an Enhanced Resistance to Cleaning-in-Place Procedures. *Foods* 8.

Ostrov, I ,.Sela, N., Belausov, E., Steinberg, D., and Shemesh, M., 2019b. Adaptation of *Bacillus* species to dairy associated environment facilitates their biofilm forming ability. *Food Microbiology* 82:316-324.

Ostrov, I., Sela, N., Freed, M., Khateb, N., Kott-Gutkowski, M., Inbar, D., and Shemesh, M., 2015. Draft Genome Sequence of *Bacillus licheniformis* S127, Isolated from a Sheep Udder Clinical Infection. *Genome Announc* 3.

Pechook, S., Sudakov, K., Polishchuk, I., Ostrov, I., Zakin, V., Pokroy, B., and Shemesh, M .2015 ,.Bioinspired passive anti-biofouling surfaces

preventing biofilm formation. *Journal of Materials Chemistry B* 3:1371-1378.

Peng, J.S., Tsai, W.C., and Chou, C.C., 2002. Inactivation and removal of *Bacillus cereus* by sanitizer and detergent. *Int J Food Microbiol* 77:11-18.

Pranting, M., Negrea, A., Rhen, M., and Andersson, D.I., 2008. Mechanism and fitness costs of PR-39 resistance in Salmonella enterica serovar Typhimurium LT2. *Antimicrob Agents Chemother* 52:2734-2741.

Ranieri, M.L., Huck, J.R., Sonnen, M., Barbano, D.M., and Boor, K.J., 2009. High temperature, short time pasteurization temperatures inversely affect bacterial numbers during refrigerated storage of pasteurized fluid milk. *J Dairy Sci* 92:4823-4832.

Ranieri, M.L., Ivy, R.A., Mitchell ,W.R., Call, E., Masiello, S.N., Wiedmann, M., and Boor, K.J., 2012. Real-time PCR detection of *Paenibacillus* spp. in raw milk to predict shelf life performance of pasteurized fluid milk products. *Appl Environ Microbiol* 78:5855-5863.

Riool, M., de Breij, A ,.Drijfhout, J.W., Nibbering, P.H., and Zaat, S.A.J., 2017. Antimicrobial Peptides in Biomedical Device Manufacturing. *Frontiers in Chemistry* 5.

Salta, M., Wharton, J.A., Stoodley, P., Dennington, S.P., Goodes, L.R., Werwinski, S., Mart, U., Wood, R.J ,.and Stokes, K.R., 2010. Designing biomimetic antifouling surfaces. *Philos Trans A Math Phys Eng Sci* 368:4729-4754.

Santhosh, S.M. and Natarajan, K., 2015. Antibiofilm Activity of Epoxy/Ag-TiO2 Polymer Nanocomposite Coatings against *Staphylococcus aureus* and Escherichia Coli. *Coatings* 5:95-114.

Sebastian, D., Yao, C.W., and Lian, I., 2018. Mechanical Durability of Engineered Superhydrophobic Surfaces for Anti-Corrosion. *Coatings* 8.

Shaheen, R., Svensson, B., Andersson, M.A., Christiansson, A., and Salkinoja-Salonen, M., 2010. Persistence strategies of *Bacillus cereus* spores isolated from dairy silo tanks. *Food Microbiol* 27:347-355.

Sharma, M. and Anand, S.K., 2002. Biofilms evaluation as an essential component of HACCP for food/dairy industry - a case. *Food Control* 13:469-477.

Simoes, M., Simoes, L.C., and Vieira, M.J., 2010. Areview of current and emerging control strategies. *LWT - Food Science and Technology* 43:573-583.

Sun, T.L., Tan, H., Han, D., Fu, Q., and Jiang, L., 2005. No platelet can adhere - Largely improved blood compatibility on nanostructured superhydrophobic surfaces. *Small* 1:959-963.

Teh, K.H., Lindsay, D., Palmer, J., Andrewes, P., Bremer, P., and Flint, S., 2013. Lipolysis within single culture and co-culture biofilms of dairy origin. *Int J Food Microbiol* 163:129-135.

Wirtanen, G., Husmark, U., and Mattila-Sandholm, T., 1996. Microbial evaluation of the biotransfer potential from surfaces with *Bacillus* biofilms after rinsing and cleaning procedures in closed food-processing systems. *Journal of Food Protection* 59:727-733.

נספח לדו"ח בנושא: פיתוח ויישום טכנולוגיה אנטימיקרוביאלית לעיכוב התבססות חיידקים במזון.

קוד הזיהוי: 421-0306-18

קוד מדען: 20-14-0027

השנה הקלנדרית עליה מוגש הדו"ח: 2018

שנת הדו"ח שלישית מתוך סה"כ 3 שנים.

שמות החוקרים:

ד"ר משה שמש, המחלקה לאיכות מזון ובטיחותו, מינהל המחקר החקלאי, מרכז וולקני.
moshesh@volcani.agri.gov.il

פרופ' בעז פוקרוי, המחלקה להנדסה ולמדע של חומרים, הטכניון מכון טכנולוגי לישראל.
bpokroy@tx.technion.ac.il

Supporting Information for the research report on subject:

Developing and application of novel antimicrobial technology mitigating bacterial establishment in food

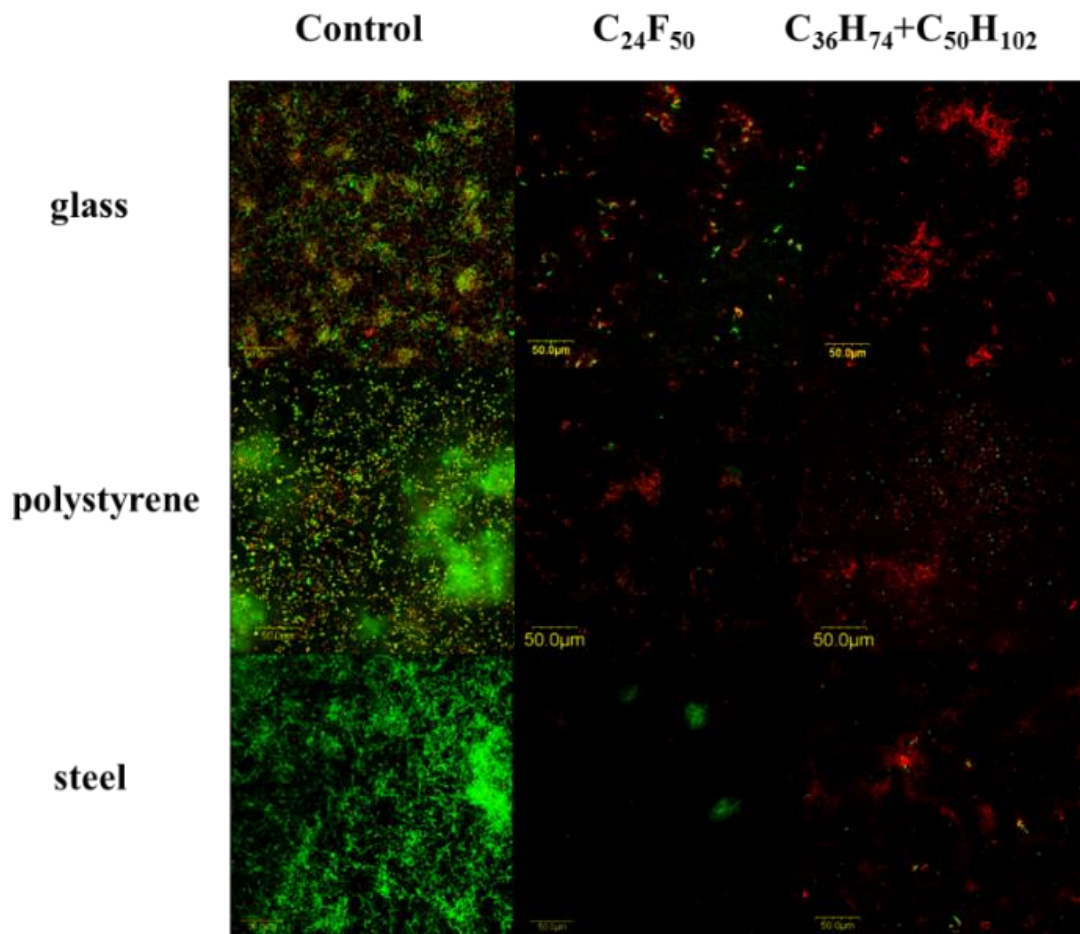

Figure S1. CLSM images of *B. paralicheniformis* S127 isolates biofilms formed on $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ polymer surfaces (before the improving the quality of the coatings by modification of the evaporation process), thermally evaporated on glass, polystyrene and stainless steel substrates, after 48 h of cultivation. Scale – 50 µm.

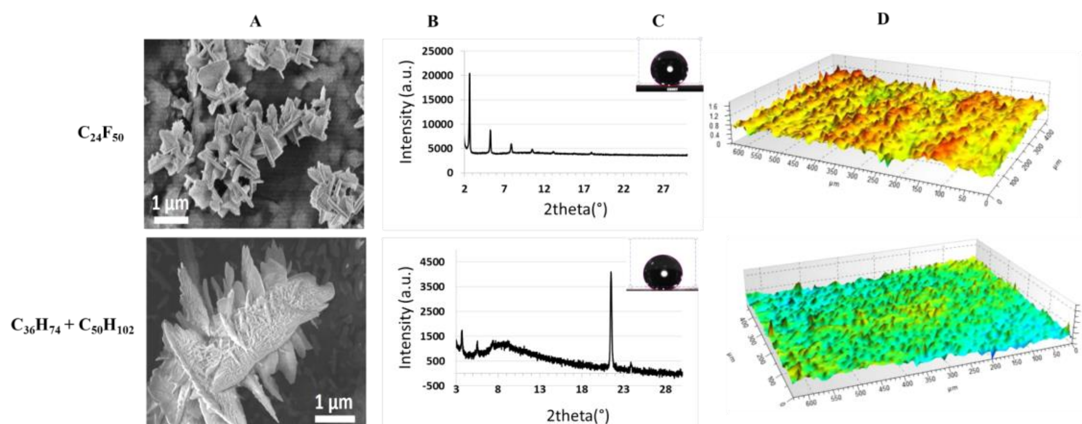

Figure S2. Complex characterization of $C_{24}F_{50}$ (top panel) and $C_{36}H_{74}$+$C_{50}H_{102}$ (bottom panel) wax surfaces, used in the previous study (before refinement). A – SEM images of wax crystals structure and distribution; B – XRD pattern collected from the tested surface; C – contact angle between water droplet and the tested wax surface; D – confocal microscopy 3D topographic images.

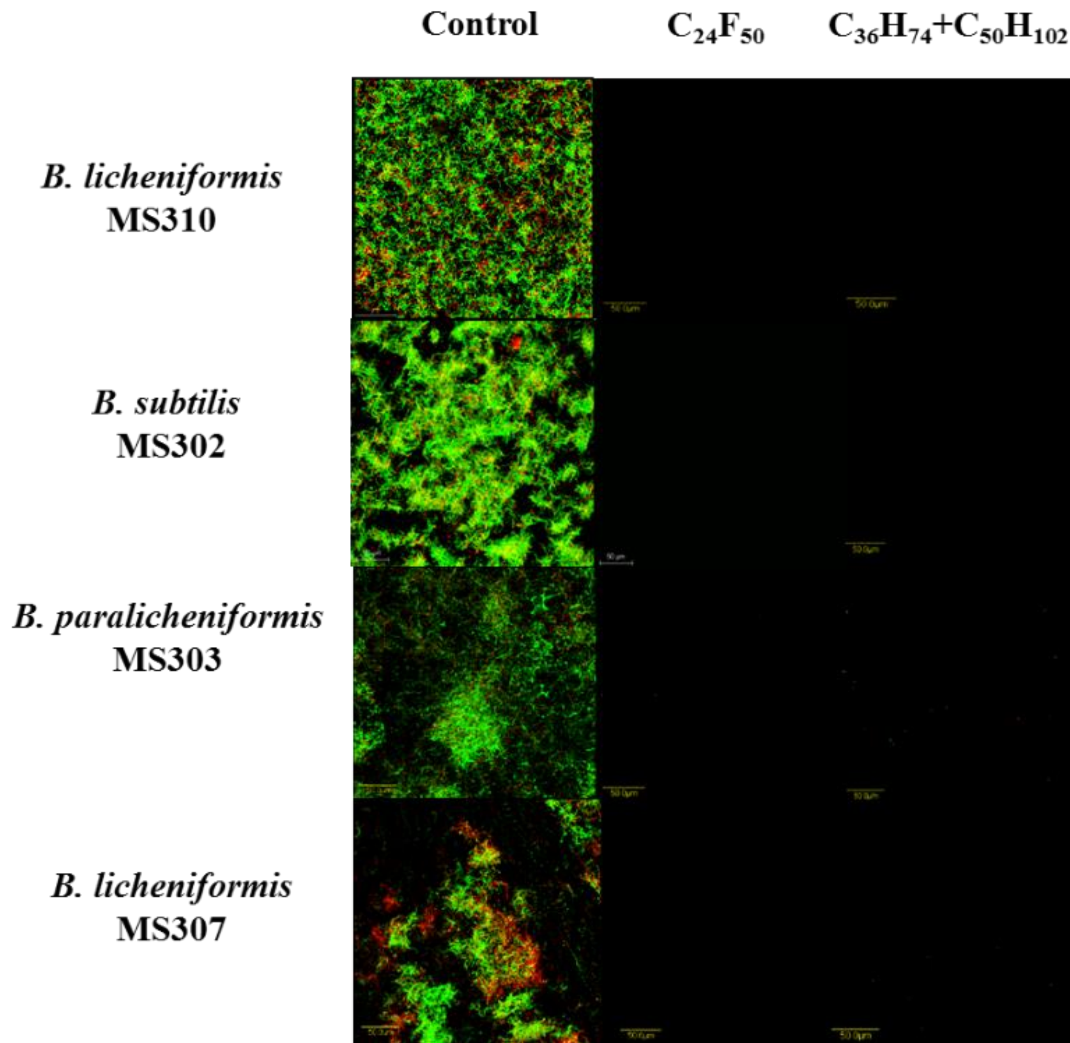

Figure S3. CLSM images of dairy *Bacillus* isolates biofilms formed on $C_{24}F_{50}$ and $C_{36}H_{74}$+$C_{50}H_{102}$ polymer surfaces, thermally evaporated on stainless steel substrates, after 48 h of cultivation. Scale – 50 μm.

Table S1. Analysis of *B. paralicheniformis* S127 biofilms formed on improved-quality $C_{24}F_{50}$ and $C_{36}H_{74}+C_{50}H_{102}$ polymer surfaces, thermally evaporated on stainless steel, glass and polystyrene substrates, after 48 h of cultivation

Biofilm depth and relative fluorescence intensity data are based on the data obtained using CLSM. The Image J program was used to analyze the relative quantities of live and dead cells in the biofilm. The results are means and SD of two different biological experimental repeats performed in triplicate.

Average biofilm depth, μm			
Treatment	Control	$C_{24}F_{50}$	$C_{36}H_{74}+C_{50}H_{102}$
Glass	50±21	8±11	17±15
Polystyrene	68±27	13±9	14±5
Steel	55±9	5±6	4±8
Relative fluorescence intensity (live/dead)			
Glass	526±265/815±786	21±6/17±11	33±12/122±126
Polystyrene	370±270/612±261	27±15/86±69	23±16/156±143
Steel	348±283/432±170	11±3/10±3	12±3/38±44

Table S2. Analysis of *B. paralicheniformis* S127 biofilms formed on improved-quality $C_{24}F_{50}$ and $C_{36}H_{74}+C_{50}H_{102}$ polymer surfaces, thermally evaporated on stainless steel, glass and polystyrene substrates, after 7d of cultivation

Biofilm depth and relative fluorescence intensity data are based on the data obtained using CLSM. The Image J program was used to analyze the relative quantities of live and dead cells in the biofilm. The results are means and SD of two different biological experimental repeats performed in triplicate.

Average biofilm depth, μm			
Treatment	Control	$C_{24}F_{50}$	$C_{36}H_{74}+C_{50}H_{102}$
Glass	32±7	0±0	6±4
Polystyrene	55±6	6±8	12±9
Steel	29±0	7±1	12±4
Relative fluorescence intensity (live/dead)			
Glass	440±295/539±180	8±7/5±5	8±0/188±216
Polystyrene	386±195/401±147	9±1/24±1	9±13/208±170
Steel	276±188/549±130	9±5/8±5	11±7/46±54

Table S3. Analysis of biofilms of dairy-associated *Bacillus* strains formed on $C_{24}F_{50}$ and $C_{36}H_{74}+C_{50}H_{102}$ polymer surfaces, thermally evaporated on stainless steel substrates, after 48 h of cultivation. Biofilm depth and relative fluorescence intensity data are based on the data obtained using CLSM. The Image J program was used to analyze the relative quantities of live and dead

Average biofilm depth, µm			
Treatment	Control	$C_{24}F_{50}$	$C_{36}H_{74}+C_{50}H_{102}$
B. licheniformis MS310	44±29	6±8	9±15
B. subtilis MS302	53±25	6±6	6±12
B. paralicheniformis MS303	47±8	0	17±24
B. licheniformis MS307	20±5	0	14±19
Relative fluorescence intensity (live/dead)			
B. licheniformis MS310	662±329/704±517	8±1/7±0	8±1/9±2
B. subtilis MS302	773±484/757±22	44±21/45±22	9±3/18±7
B. paralicheniformis MS303	567±45/407±152	8±1/9±0	8±1/17±2
B. licheniformis MS307	435±50/190±73	29±30/11±5	30±15/10±7

cells in the biofilm. The results are means and SD of two different biological experimental repeats performed in triplicate

Table S4. Analysis of biofilms of dairy-associated *Bacillus* strains formed on $C_{24}F_{50}$ and $C_{36}H_{74}+C_{50}H_{102}$ polymer surfaces, thermally evaporated on PTFE substrates, after 24 h of cultivation in SM in the CDFF. Biofilm depth and relative fluorescence intensity data are based on the data obtained using CLSM. The Image J program was used to analyze the relative quantities of live and dead cells in the biofilm. The results are means and SD of two different biological experimental repeats performed in triplicate.

Average biofilm depth, μm			
Treatment	Control	$C_{24}F_{50}$	$C_{36}H_{74}+C_{50}H_{102}$
B. paralicheniformis S127	35±12	0±0	0±0
B. licheniformis MS310	54±16	0±0	0±0
B. subtilis MS302	49±20	0±0	0±0
B. paralicheniformis MS303	45±12	0±0	0±0
B. licheniformis MS307	39±14	0±0	0±0
Relative fluorescence intensity (live/dead)			
B. paralicheniformis S127	1330±905/745±34	7±0/7±0	8±0/7±0
B. licheniformis MS310	953±394/304±50	7±0/7±0	7±0/7±0
B. subtilis MS302	1950±633/233±73	7±0/6±0	8±0/6±0
B. paralicheniformis MS303	1586±287/661±96	8±0/7±0	7±0/6±0
B. licheniformis MS307	19062±47/229±80	8±0/7±0	7±0/7±0