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Biofilm formation capacity of *Bacillus cereus* on silicone, polyethylene terephthalate, Teflon, and aluminium food contact materials

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ABSTRACT

Biofilms on food contact materials represent public health issues because they are resistant to cleaning and disinfection. This study aims to assess the *Bacillus cereus* biofilm formation capacity on silicone, polyethylene terephthalate, Teflon, and aluminium food contact materials. The biofilm biomass was analysed with the crystal violet assay method. We used the standard strain *B. cereus* CCM 2010, wild strain *B. cereus* 100 and spores of those two strains. The results show that both the vegetative form the bacteria and it spores form large amounts of biofilm on silicone, followed by polyethylene terephthalate, Teflon, and aluminium. More detailed analysis has shown that spores form more biomass on all materials in comparison to the vegetative form and that the standard strains form low levels of biofilm in contrast to the wild strains. Selecting proper material with the lowest biofilm formation potential can prevent or reduce food contamination and consequently increase food safety.

Key words: biofilm; *Bacillus cereus*; food contact materials

POVZETEK

Biofilmi na kontaktnih površinah predstavljajo pomemben javno zdravstveni izziv, saj so bolj odporni na čiščenje in dezinfekcijo kot planktonske celice. Namen raziskave je bil ovrednotiti količino biofilma na materialih za stik z živili, kot so silikon, polietilen tereftalat, Teflon in aluminij. Količina biomase biofilma na površini je bila ocenjena z metodo kristal vijolično. V raziskavi smo uporabili standardni sev *B. cereus* CCM 2010, divji sev *B. cereus* 100 in spore obeh omenjenih sevov. Rezultati kažejo, da tako vegetativna oblika, kot spore obeh sevov tvorijo velike količine biofilma na silikonu, sledi mu polietilen tereftalat, teflon in aluminij. Bolj natančna analiza kaže, da spore tvorijo več biomase na vseh materialih v primerjavi z vegetativno obliko ter da standardni sev *B. cereus* tvori manj biofilma v primerjavi z divjim sevom. Izbira primernega materiala z najmanjšim možnim potencialom za nastanek biofilmov lahko zmanjša ali prepreči kontaminacijo živil in posledično izboljša varnost.

Ključne besede: biofilm; *Bacillus cereus*; materiali za stik z živili

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INTRODUCTION

Most household food contact materials are in permanent contact with foodstuff; therefore, the probability of acquiring surface contaminants from contact materials into the food is high [1]. The contamination of food contact surfaces during food handling due to bacteria present in foodstuff is one of the main causes of alimentary intoxication [2]. Biofilm formation is a biological phenomenon as bacteria tend to live on surfaces rather than in a planktonic state. When embedded in a biofilm, cells are protected against harsh environmental conditions, such as chemicals, physical stresses, and antimicrobial agents, because their exopolysaccharide matrices act as protective barriers that limit penetration into the biofilm [3]. Recent foodborne outbreaks have focused on biofilms on food contact materials, examining the sources of food contamination [4]. The most commonly used materials in household environments are wood, ceramics, glass, different types of metals, silicones, Teflon, and polyethylene terephthalate [5]. Those materials are used for kitchenware, such as bottles, jars, tubs, models for baking and freezing, pastry brushes, lids, pots, pans, containers, wrappings, baking sheet, milk jugs and others. *B. cereus* is a gram-positive microorganism which can form spores under harsh environmental conditions. They are pathogenic, facultative anaerobic bacteria that produce toxins. Some vegetative strains are harmful to humans and cause foodborne illness, including nausea, vomiting, and diarrhoea [6]. *B. cereus* is a pathogenic bacterium that is frequently found in various types of raw and cooked foods, and its ability to survive high cooking temperatures requires that cooked foods be served hot or cooled rapidly to prevent the growth of this bacterium [7]. Because of its ability to form highly resistant spores and its natural spread in the wild, *B. cereus* is a major food safety concern. The spores are common in soil and spread easily to cows' udders and from there to the raw milk. In addition to the ability to survive pasteurization, they also attach very well to most household materials [8] from which they can spread throughout the kitchen environment. It is well known that *B. cereus* in vegetative cells or spores tends to adhere to rough surfaces [9, 10]. One reason for this can be the presence of appendages, proteins, polysaccharides, and lipids that allow attaching and consequently forming the biofilm [11]. Moreover, some authors have reported that the surface energy of *B. cereus*, which is highly hydrophobic, is able to adhere firmly to various materials such as those found during food processing in household environments [12]. A more specific study by Ekman et al. [13] demonstrated a transfer of *B. cereus* from paper surfaces to foods. Similarly, Le Gentil et al. [14] analysed the attachment and detachment of *B. cereus* in cleaning processes and found that re-attachment can be a reason for surface contamination. Furthermore, Fink et al. [6] reported that the removal of *B. cereus* from polyurethane conveyor belts with industrial cleaning agent is difficult if not impossible. The persistence of microbial biofilms represents a significant challenge to the establishment and maintenance of hygienic conditions in different environments. The possibility of bacterial

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Four different food contact materials that are often used in the home kitchen environment were tested for *B. cereus* biofilm formation: aluminium, silicon, Teflon, and poliethylenetherephalate (PET).

multiplication in foods after storage and/or handling must be taken into account when defining safe levels for human consumption [15]. The objective of this study was to analyse the capacities of *B. cereus* biofilm formation on silicone, polyethylene terephthalate, Teflon, and aluminium food contact materials and to provide consumer information on material hygiene.

METHODS

Bacteria, growth and sporulation media

In the experiment, wild strain *B. cereus* 100 (isolated from milk and kept at the University of Ljubljana, Faculty of Health Sciences), standard strain *B. Cereus* CCM 2010 (Czech Collection of Microorganisms, Brno, Czech Republic), and spores of these two strains were used.

Methods

In this study, four different food contact materials that are often used in the home kitchen environment were tested for *B. cereus* biofilm formation: aluminium, silicon, Teflon, and poliethylenetherephalate (PET). The materials were cut into the coupons of 10 × 10 mm, which were washed with 98% ethanol (Sigma-Aldrich, Misuri, ZDA) and destilled water and dried before being autoclaved. An Olympus CX40 optical microscope with an off-the-bench illuminator and CCD CMOS camera (Camera Digital microscope Electronic Eyepiece for Image) was used to visualize the structures of the materials (Figure 1). The surface roughness of the selected material was determined by mechanical profilometer Form Talysurf Series 2 from Taylor-Hobson Ltd., Leicester, Great Britain.

Determining the biofilm biomass formation capacity

Staining biofilm biomass remains a useful baseline technique to provide a practical, inexpensive, and reliable method for the detection of biofilms.

To determine the biofilm's biomass formation, a modified method by Bohinc et al. [1] and Kubota et al. [16] was used. Staining biofilm biomass remains a useful baseline technique to provide a practical, inexpensive, and reliable method for the detection of biofilms [17]. Bacteria from the collection were transferred on the nutrient agar and incubated at 37 °C 24h. After that, a single colony of strain was transferred from the nutrient agar to the nutrient broth (Biolife, Italy) and incubated under the same conditions. Next, the bacterial culture was diluted in a 1:300 ratio, with fresh nutrient broth. Sterile coupons were transferred in a sterile petri dish and exposed to the bacterial suspension; 4 mL of the nutrient broth with bacterial cultures in a ratio of 1:300 was added. The bacterial suspension and coupons were incubated for 24 hours at the temperature of 37 °C. After the incubation time, the bacterial suspension was removed and the coupons were rinsed three times with phosphate buffered saline (PBS) (80 g of NaCl, 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4 in 1 L) to remove unattached or loosely attached cells. The coupons with adhered bacterial cells were exposed to 3 mL 0.1% (w/v) crystal violet suspension (Merck, Germany) for 5 min. Then the coupons were rinsed three times with the PBS

buffer to remove excess dye. In the next step, the dye was extracted from the cells with 200 μL 96% ethanol. The optical density (OD) of the ethanol/dye solution was measured with an Infinite 200® PRO microplate reader (Tecan, Austria, GmbH) at the wavelength of 620 nm (Figure 1).

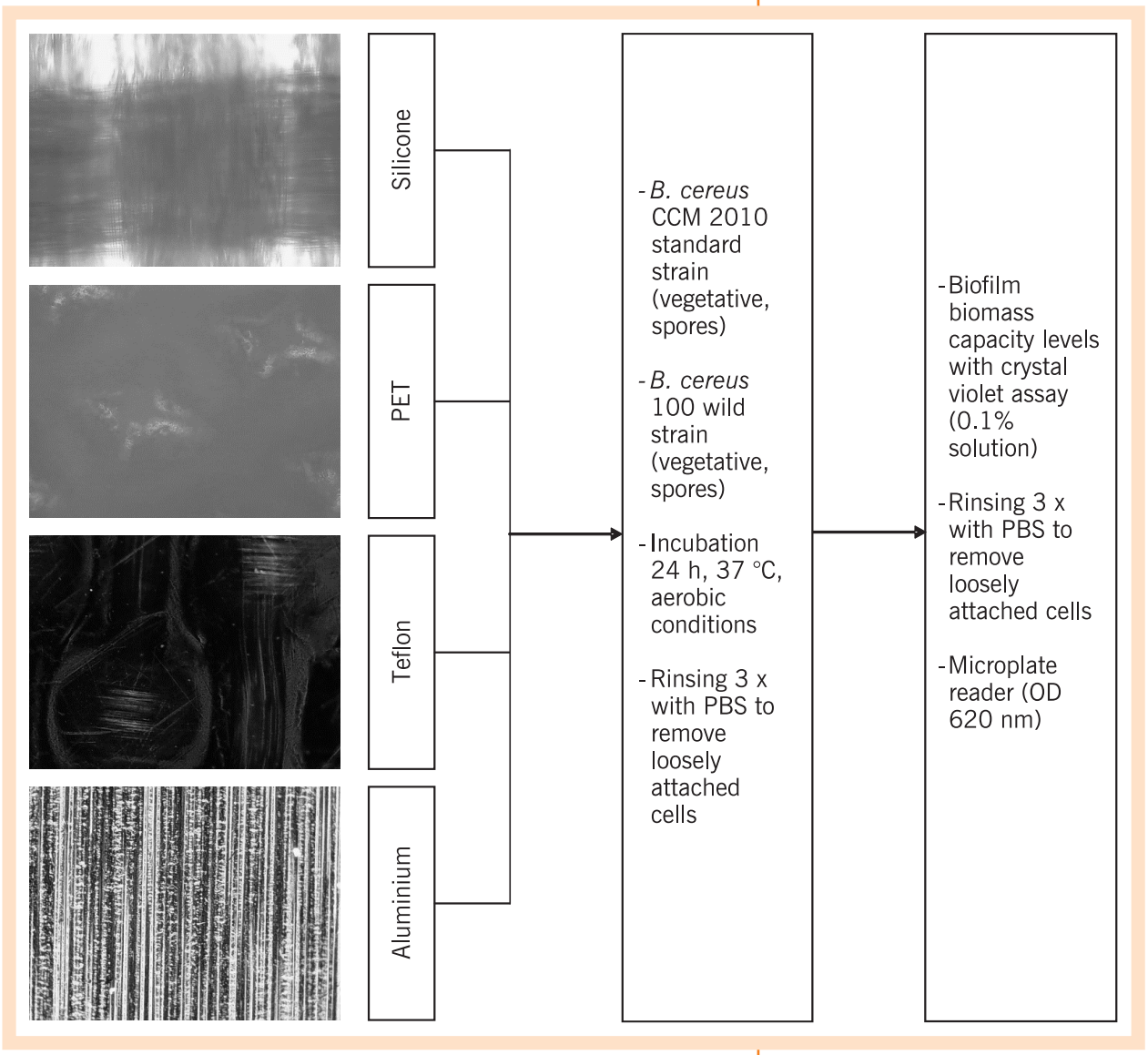

Figure 1.
B. cereus biofilm capacity assessment process flowchart.

All the experiments were performed with five parallels and three repetitions. For assay of the spores biofilm, the sporulation Casein-Casein-Yeast (CCY) medium (Sigma-Aldrich, USA) was used. The method of spore production was introduced by Abbas et al. [18] and modified as follows. To obtain spores form vegetative cells, both bacterial strains were incubated in a CCY medium for 24 hours. In the next step, bacterial culture was centrifuged with 4000 × g for 10 minutes to separate the cells from the liquid medium. The cells were re-suspended with a PBS buffer. The process was repeated three times to remove the entire liquid medium. At the final step of the culture process, the suspension was exposed to 80 °C for 10 min to destroy the

Several studies indicate that the materials of kitchen accessories (e.g. cutlery, knives, and chopping boards) represent a high risk for bacterial cross-contamination.

remaining vegetative cells. To determine the quantity of *B. Cereus* spores biofilm, the same procedure as for the vegetative form of *B. cereus* described above was used.

Statistical analysis was provided using R software version 3.1.3 and a Student's t-test comparing the OD of crystal violet dye released from the biofilm regarding the form and material. The statistical significance was set to $p < 0.05$.

RESULTS AND DISCUSSION

Food contact materials are the main source of alimentary intoxication in the domestic environment. Several studies indicate that the materials of kitchen accessories (e.g. cutlery, knives, and chopping boards) represent a high risk for bacterial cross-contamination [19]. The results of material characterization show that PET has the highest roughness of 1.2 μm, followed by silicone with 0.9 μm, Teflon 0.4 μm and aluminium with 0.2 μm. The results show that *B. Cereus* standard and wild strains, the vegetative form, and spores grow on all analysed food contact materials. The results show the least biofilm biomass on aluminium surfaces and the highest amounts on silicone (Figure 2). Furthermore, the biofilm formation capacity for standard strain *B. cereus* CCM 2010 initially inoculated from vegetative cells shows, on average, the highest biofilm capacity for silicone, followed by PET, Teflon, and aluminium (Figure 2a). Similar results can be obtained for standard strain *B. cereus* CCM 2010 inoculated from spores, for which abundant biofilm formation was found on silicone, but the fewest spores on aluminium (Figure 2b). The wild strain of *B. cereus* 100 vegetative cells formed high biofilm biomass on silicone, PET, aluminium but much less biomass was found on Teflon (Figure 2c). Complementary to that, *B. cereus* 100 wild strain biofilm inoculated from spores show the highest biofilm formation on silicone, followed by PET and aluminium. The lowest amount of biofilm inoculated from spores was found on Teflon (Figure 2d). This demonstrates that, generally (apart from PET), total amounts of biofilm biomass correspond to material roughness. It is generally accepted that the smoother the surface is, the lower the number of adhered cells is present [1, 20]. More importantly, this study indicates that a significant difference in total biofilm biomass exists when comparing material, bacterial strain, and form (vegetative form or spores).

B. cereus 100 wild strain biofilm inoculated from spores show the highest biofilm formation on silicone, followed by PET and aluminium. The lowest amount of biofilm inoculated from spores was found on Teflon.

Shaheen et al. [21] studied adhesion potential of different strains of *B. cereus* and found that spores adhere to the surface more firmly than vegetative cells do. Similar results were presented by Kolari et al. [22], who reported that hydrophobic spores of *B. cereus* are the most adhesive, one reason for which can be that strong adhesion makes favourable conditions for the spread of spores with rinse water from one location to another. Exosporium plays a significant role in spore interaction with materials, probably by providing a larger contact surface with materials. Kumariand Sarkar [23] reported that the strong adhesion potential of *B. cereus* spores has been attributed to the

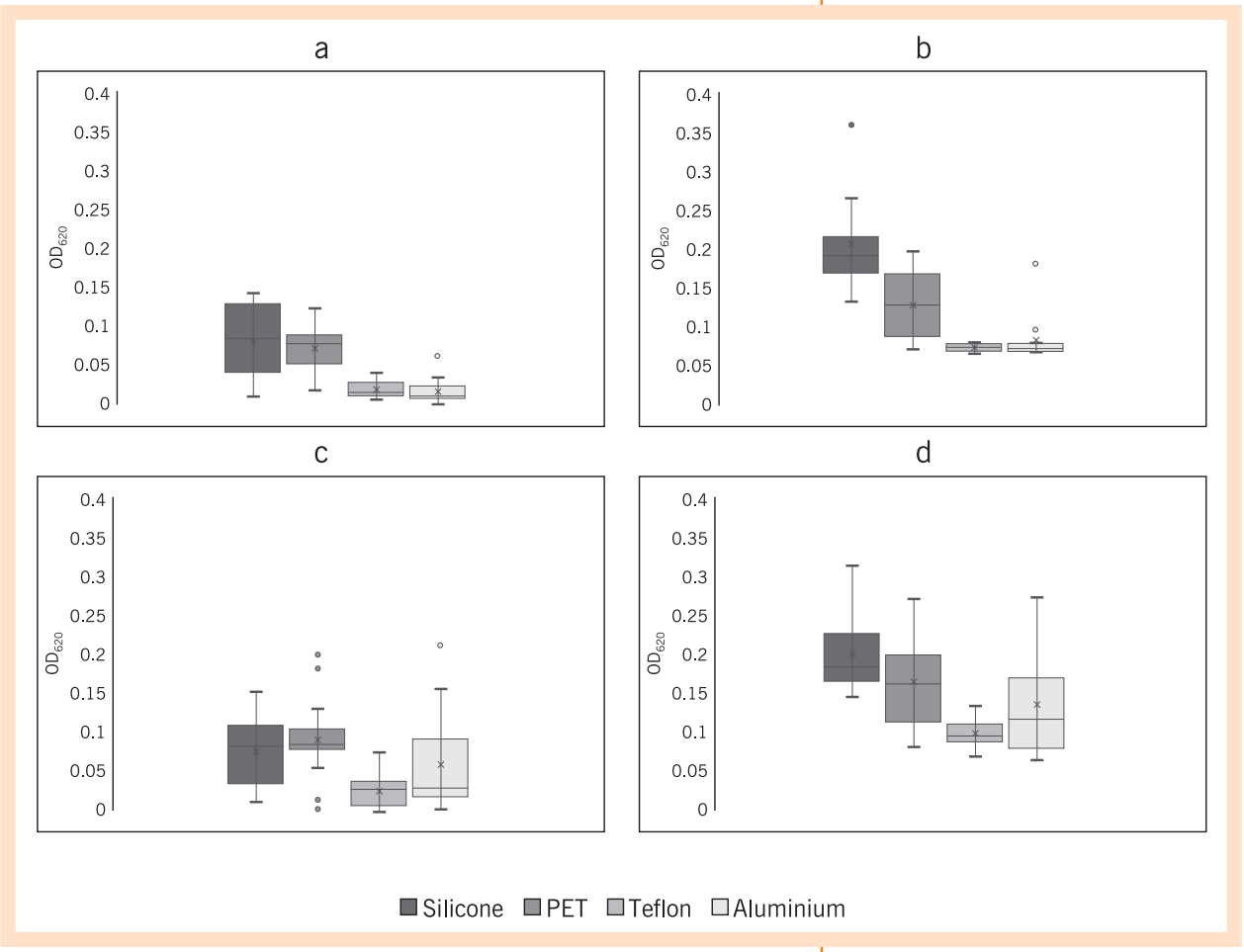

Figure 2.
Optical densities (mean, quartiles, min and max) of released crystal violet dye from *B. cereus* biofilm inoculated from vegetative form (a, b) and spores (c, d) on silicone, PET, Teflon, and aluminium.

hydrophobic character of exosporium, which varies between strains. We also found that wild strain *B. cereus* biofilm causes more biomass growth on all material in comparison to the standard strain. Comparable to our study, Hayrapetyan et al. [3] analysed standard and the undomesticated food isolate strain *B. cereus* and found significant differences in OD after 24 hours of incubation on stainless steel surfaces. Similar to that, other researchers [24, 25] reported that the amounts of biofilm biomass can vary between the strains of the same species.

Comparison of optical densities of released crystal violet dye from biofilm biomass reveals statistically significant higher optical densities for biofilm inoculated from spores on all materials and both strains ($p < 0.05$). The most abundant differences between biofilm inoculated from vegetative form and spores can be observed for silicone, in the case of both strains (ΔOD *B. cereus* CCM 2010 = 0.1317; ΔOD *B. cereus* 100 = 0.1220). In contrast, the smallest difference between biofilm inoculated from vegetative form and spores was found for Teflon when comparing the standard strain *B. cereus* CCM 2010 (ΔOD = 0.521) and the wild strain *B. cereus* 100 (ΔOD = 0.068) (Table 1).

Wild strain *B. cereus* biofilm causes more biomass growth on all material in comparison to the standard strain.

Table 1. Comparison of optical densities of crystal violet dye released from B. cereus biofilm inoculated from vegetative form and spores on silicone, PET, teflon, and aluminium.

Material	*B. cereus*	OD_{620} vegetative form	OD_{620} spores	Δ OD (/)	t-value	p-value
Silicone	Standard strain CCM 2010	0.0810	0.2127	0.1317	11.925	0.000008**
PET		0.0712	0.1438	0.0726	10.367	0.000006**
Teflon		0.0188	0.0709	0.0521	23.969	<0.000000**
Aluminium		0.0163	0.0805	0.0642	7.396	0.000049**
Silicone	Wilde strain 100	0.0778	0.1998	0.1220	9.278	0.000003**
PET		0.0921	0.1625	0.0704	8.137	0.000005**
Teflon		0.0280	0.0968	0.0688	34.32	<0.000000**
Aluminium		0.0613	0.1343	0.0730	4.085	0.001805*

Legend: * $p<0.05$; ** $p<0.000$

CONCLUSIONS

The selection of proper material with the lowest adhesion potential, along with cleaning procedures and good hygiene behaviour, represents the primary strategy for decreasing the risks of food poisoning in household environments. The results of our study demonstrated that aluminium and Teflon have much lower biofilm capacity in comparison to others. Moreover, the results of our study indicate that biofilm biomass formation depends not only on material properties but also on bacterial strain and form. By understanding the relationship between material surface properties and bacterial adhesion, strategies can be developed that would greatly inhibit, if not prevent, biofilm growth in domestic environments.

The selection of proper material with the lowest adhesion potential, along with cleaning procedures and good hygiene behaviour, represents the primary strategy for decreasing the risks of food poisoning in household environments.

Biofilm biomass formation depends not only on material properties but also on bacterial strain and form.

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