Impact of *Lactococcus lactis* spp. *lactis* Bio Adhesion on Pathogenic *Bacillus cereus* Biofilm on Silicone Flowing System

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Received: 24 October 2012 / Accepted: 12 February 2013 / Published online: 22 February 2013
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Abstract *Bacillus cereus* is a food pathogen that can attach on most of the surfaces and form biofilms, which facilitate the persistence and resistance toward antimicrobials. The aims of this study were (i) to characterize the structural dynamics of *B. cereus* sessile growth in two nutritional environments (with or without a nutrient flow), and (ii) to evaluate the impact of bio adhesion of *Lactococcus lactis* on *B. cereus* biofilm. Significantly greater biofilm volume and thickness were observed under dynamic conditions than under static conditions after 48 h and *B. cereus* biofilm was highly organized. The variation of physico-chemical characteristics of silicone by *B. cereus* bio adhesion favours the adhesion of hydrophilic *Lc. lactis* on the surface adhered by biofilm. *Lc. lactis* was able to adhere to silicone surface and produce biofilm obviously exhibited a significant reduction of *B. cereus* adhered cells up to nine orders of magnitude after 48 h of contact with competitive activity for nutrient and oxygen. This study constitutes a step ahead in developing strategies to prevent microbial colonization of silicone with lactococcal protective biofilm.

Keywords *Bacillus cereus* · Biofilm · ESEM · Flow cytometry (FCM) · *Lactococcus lactis* · Adhesion · Protective biofilm

Introduction

Microbial adhesion to surfaces is the onset of the development of a biofilm. After an initial step of reversible, then irreversible adherence, bacteria grow as microcolonies and spread on the surface [1]. Biofilms can become a health hazard by harboring pathogenic bacteria such as *Bacillus cereus*, a spore former is still an important gram-positive pathogenic bacteria, and numerous studies have shown that these bacteria is capable of attaching and developing biofilms on a variety of surfaces, such as stainless steel [2]. Several ways to study biofilms have been used to identify and characterize the bacterial elements and genetic determinants involved in biofilm development. For instance, plate counting has been used to quantify sessile cells of gram-positive bacteria on abiotic surfaces [3]. Also, biofilms of *B. cereus* have been quantified by using the microplate adhesion method [4], while their structures have been investigated by scanning electron microscopy (SEM) [5], or laser-scanning confocal microscopy (LSCM) [6]. Chemistry characterization of surface by goniometry study [7, 8] was recently developed and used in biofilm interaction on substratum. Physico-chemical interactions can be classified into three classes: Lifshitz van der Waals interactions, electrostatic interactions [9] and polar or Lewis acid–base interactions [10]. Alternative short-term approaches are therefore needed to provide quantitative and kinetic analyses to characterize initial bacterial cell surface attachment. The multi-parametric nature of Flow cytometry (FCM) introduced by Beloin et al. [11], offered the opportunity of correlating initial adhesion with surface property changes and free-floating cell aggregation shifts, which are two phenomena involved in surface colonization. Nowadays, protective biofilm formation of food industry surfaces or medical devices can be beneficial against

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adhesion of the undesirable planktonic microorganism [12]. In recent years, biofilms of lactic acid bacteria have received considerable attention for their potential use in the settlement of a competitive flora [13, 14] and changes of cell surface physico-chemical properties increased adhesion of protective biofilms. In the present study, we investigated the development of *B. cereus* in biofilms by environmental scanning electron microscopy (ESEM) and Flow cytometry (FCM) under environmental static and flowing conditions. Second, we determined the impact of *Lactococcus lactis* bio adhesion on *B. cereus* biofilm.

Materials and Methods

Bacterial Strain and Growth Conditions

A strain of *B. cereus* was isolated from biofilm taken from dairy processing line of the collect centre in north of Tunisia. The strain has been previously identified by PCR-sequencing targeting 16S rDNA genes. Following BLAST analysis, strains was affiliated to *B. cereus* R13 (97 %) (NCBI Blast software). Frozen *B. cereus* had been transferred in Nutrient broth (Difco, USA) and subcultured in 10 mL BN broth at 30 °C for 18 h before the cells were used. The culture was harvested by centrifugation for 15 min at 8,400×g, and washed twice and resuspended in 0.1 M KNO₃ solution.

Characterization of *Lactococcus lactis* Surface

A suspension of cells in KNO₃ solution was deposited into a 0.45 µm cellulose acetate filter by first washing the filter with 10 mL of distilled water for wetting, and then 10 mL of the cell suspension was added obtaining a thick lawn of cells after filtration by means of negative pressure. Hydrophobicity and surface free energy characteristics were inferred from measured contact angles using a goniometer (GBX instruments, France) by the sessile drop method [8].

Biofilm Growth Conditions

Static Biofilm Experiments

The silicone surface, was cut into 1 cm² squares with 2 mm. Coupons were first cleaned by washing them with neutral liquid detergent and water, followed by rinsing with distilled water, air-dried, and sterilized at 121 °C for 15 min [6]. The cleaned and sanitized coupons were inserted in separate 55-mm-diameter petri dishes that contained 20 mL of culture of *B. cereus*, which were incubated at 37 °C. The medium was removed after 2 h,

and then every 24 h by fresh culture of *B. cereus* (20 mL) was added. The initial number of cells was ~10⁶ cfu/mL. Cell adhesion and biofilm development were evaluated after 3, 24, and 48 h at 37 °C in a shaker rotating at 120 rpm. Following incubation, the medium was removed and 10 mL of saline solution (0.9 % NaCl) was gently poured onto the coupons to remove loosely adhering bacteria. Negative control was obtained by placing the coupon in a saline solution without bacterial cells. Afterward, each coupon was placed into Petri plates and analyzed for ESEM and FCM.

Flow-Silicone System Biofilm Experiments

Biofilm was cultivated in continuous-flow silicone system (total length 1 m, diameter 1 cm). Flow system was inoculated with overnight cultures of *B. cereus* adjusted to a concentration of 10⁶ cfu/mL. After inoculation, the medium flow was stopped for 1 h to allow bacterial adhesion, and thereafter the medium was pumped through the flow cells at 1.3 mL/min by using a peristaltic pump (ISMATEC ID 871). Biofilm development was assayed at 3, 24, and 48 h by analysis of 1 cm² silicone sections.

Application of *Lc. lactis* on Silicone Adhered by *B. cereus* Biofilm

Cells of *Lc. Lactis* were prepared with overnight shaking on MRS and were harvested by centrifugation at 6,000×g for 10 min. The cell pellets were then quickly washed twice and resuspended in sterile saline water (0.9 %). The harvested cells were adjusted to a concentration of 10⁶ cfu/mL and the system with 55 h biofilm was inoculated. The analysis of 1 cm² of silicone section was carried out after 3, 24 and 48 h of contact.

Assessment of Biofilm Viable Cells: *B. cereus* and *Lc. lactis*

The number of bacterial cells of *B. cereus* and *Lc. lactis* adhered to the silicone was determined after 3, 24 and 48 h. Initially, 1 cm² of silicone was immersed three times in 5 mL of sterile saline water (0.9 %), to remove the planktonic cells, followed by the removal of the adhered cells using sterilized swabs. The swabs were transferred to test tubes containing 10 mL of saline solution and stirred vigorously for one minute. For each measuring period, two randomly collected coupons were used as replicates [15]. Aliquots of 100 µL of each dilution were inoculated in Petri dishes containing Nutrient agar and MRS, using the (i) spread plate technique. Afterwards, (ii) microscopic observations and (iii) flow cytometry analysis have been realized.

Environmental Scanning Electron Microscopy (ESEM) Analysis

Different specimens of silicone were gently washed by sterile distilled water three times and image acquisition was performed on Scanning Electron Microscopy (ESEM Quanta 200) equipped with a tungsten filament (FEI) without fixation of cells [5].

Flow Cytometry Study

Fluorescent Probes and Staining Protocols Sessile cells were stained with a carboxyfluorescein diacetate (cFDA) (Sigma-Aldrich, USA) solution and propidium iodide (PI) (Fluka, USA), cFDA and PI stains penetrates in live and dead cells respectively. Before staining, silicone sections were diluted in Chemsol B4 buffer (AES-Chemunex, France). Live and dead assays were done by dual staining of each sample to differentiate viable, dead and stressed cells. 1 mL of the diluted suspension was firstly incubated with 10 μ L of PI (1.496 mM in distilled water) for 20 min at 37 °C. 10 μ L of cFDA (0.0217 mM in acetone). After were then added and incubation took place for 10 min at 37 °C. After short centrifugation at 14,000 $\times g$ for 1 min, cell pellets were resuspended in 1 mL of Chemsol B4 buffer before analysis by flow cytometer. Unstained cells which were preliminary not treated or incubated, during a fixed period, were analyzed by flow cytometry and used as negative controls [16].

Flow Cytometry Conditions and Data Analyses Flow cytometry analyses were performed using a Becton–Dickinson flow cytometer system can be easily upgraded to address emerging automation or sorting requirements in a laboratory (Facsclibur, BD Biosciences, USA). The cF (maximum excitation wavelength of 492 nm and an emission wavelength of 517 nm) and PI-labeled (maximum excitation wavelength of 535 nm and an emission wavelength of 617 nm) populations were spatially discriminated in dot plots of FL1 (Cf fluorescence: x-axis) and FL2 (PI fluorescence: y-axis) [16]. Two different acquisitions were performed on each sample to collect either the bacterial signal. The first acquisition was located in the lower left quadrant corresponding to unstained debris. The second acquisition corresponding to PI-labeled cells appeared on the FL2 detector in the upper left quadrant corresponded to dead cells. The third acquisition corresponding to cF-labeled cells appeared in the lower right quadrant and included viable cells. Finally, a double stained population, observed in the upper right quadrant, represented injured cells.

Statistical Analysis

Two independent experiments with two replicates each were made for the assessment of *B. cereus* biofilm development and the application of *Lc. lactis* in flow system, in order, at each sampling time. The statistical analysis was conducted by averages, determination of variances and gaps type using the ANOVA test: DATASET1.ISD by Graph Pad in stats demo version 3.0 Software. Differences were considered statistically significant threshold $p < 0.05$.

Results

Biofilm Formation Under Static and Dynamic Conditions

The sessile growth of *B. cereus* was monitored under static and flowing conditions using plate count numeration, ESEM and FCM. Under static conditions, after 3 h of contact, a high number of cells were scattered on the surface exceeding $\log_{10}$ 5.96 cfu/cm² (Fig. 1). After 7 h of growth, the rate of adhered cells increased exponentially over $\log_{10}$ 7.46 cfu/cm². After 24 h, a rate increased to $\log_{10}$ 8.85 cfu/cm². The ESEM image stacks and Matlab® analysis evidenced a uniform biofilm that can be described as organized multicellular layers of cells covering the surface (Fig. 2a). The number of adhered cells growing under dynamic conditions was different (Fig. 1). Indeed, the biofilm's volume increased during incubation irrespective of the hydrodynamic conditions with a significant increase after 24 h, and the differences were significant after 48 h. In fact, after the initial adhesion of *B. cereus*, the biofilm developed as a complex structure in which dense, ball-shaped microcolonies separated by poorly colonized zones were observed after 3 h of contact (Fig. 2). These structures were thicker after 24 and 48 h of incubation to attempt $\log_{10}$ 9.16 and $\log_{10}$ 11.35 cfu/cm² respectively (Fig. 1). Observations under a stronger magnification and Matlab® analysis showed that a silicone surface was covered by a uniform and compact biofilm (Fig. 2). Moreover, FCM showed the evolution of the physiological state of *B. cereus* cells aggregate depending on the period of contact to silicone (Fig. 2b). In fact, after 48 h, an increase of live cells level up to 91 % has been shown.

Impact of the Bio Adhesion of *Lc. lactis* on *B. cereus* Biofilm

The effect of *Lc. lactis* has been assessed by different methods. Firstly, enumeration of cells after 48 h of *Lc. lactis* application, revealed a significant reduction of

Fig. 1 Biovolume of *B. cereus* biofilm under static conditions (gray line) and under flowing conditions (black line) after 3, 24, and 48 h of incubation

B. cereus biofilm up to nine orders of magnitude (Table 1). Secondly, ESEM micrographs have revealed a haze-like film covering the *B. cereus* microcolonies on silicone after 48 h that could be corresponding to *Lc. lactis* biofilm (Fig. 3a). Indeed, after 48 h of contact, Matlab® analysis, revealed of a high rate of decrease to attempt 80 % of totally adhered surface. In other hand, from FCM pannels, we observed the formation of cell aggregates that produced higher scattering and a fluorescence signal defining a new gate. Indeed, significant bacterial cell death was observed within the culture after incubation, as indicated by the presence of noticeable number of red-labeled bacteria on left quadrant (Fig. 3b). Furthermore, PCR 16s rDNA amplification and sequencing of the microbial communities adhered to the silicone revealed the preferentially colonized by *Lc. lactis* strain after 48 h. ESEM observations and the loss of viability observed by FCM, hypothesis that *Lc. lactis* was able to adhere to silicone and produce biofilm. Bio adhesion of *Lc. lactis* may exhibit an immediate bactericidal activity, able to remove *B. cereus* biofilm.

Physico-Chemical Characterization of *Lc. lactis* Surface

Hydrophobicity, surface tension parameters and the surface energy were evaluated through contact angle measurements and calculation using the van Oss approach with applying the data to the extended-DLVO theory. Water contact angle can be used as a qualitative indication of the surface material hydrophobicity, with higher values indicating a more hydrophobic surface (θ_w (°) > 65). As it can be seen, the water contact angle obtained for *Lc. lactis* surface is hydrophilic θ_w (°) = 12.6 (Table 2). Also, *Lc. lactis* showed positive value of free energy $\Delta G_{wi} = 30.95 \text{ mJ/m}^2$ and so, can be considered hydrophilic (Table 2). In what concerns surface energy

components, *Lc. lactis* strain predominantly showed electron donation, with higher values of electron donor parameters ($\gamma^- = 54.1 \text{ mJ/m}^2$) compared to the low values of the electron acceptor parameters ($\gamma^+ = 1.5 \text{ mJ/m}^2$) (Table 2).

Discussion

The dynamics of *B. cereus* biofilm formation can vary in response to environmental conditions such as nutrient limitation, flow rate and pressure [17]. In this study, we used enumeration and ESEM to characterize and compare *B. cereus* biofilms grown under two environmental conditions. Significantly greater biofilm volume was observed under flowing conditions than under static conditions. Indeed, this result is consistent with Rieu et al. [6] demonstrated that the model used to grow *Listeria monocytogenes* biofilms deeply affects their structure and rate of adhered cells. Usually, the hydrodynamic conditions can be highlighted by the increased of fluid flow towards or parallel to a substratum surface results in faster adhesion of microorganisms due to higher mass transport despite the presence of higher fluid shear stimulating their detachment [18]. Afterwards, the impact of *Lc. lactis* bio adhesion on silicone with adhered *B. cereus* has been assessed at different time of contact. Results showed that *Lc. lactis* was able to adhere to silicone surface and produce biofilm exhibited a significant reduction of *B. cereus* adhered cells after 48 h. In fact, our result was corroborated with García-Almendárez et al. [19] showed the competitive exclusion of *Listeria* by *Lc. lactis* UQ2 and reported that can be due to the ability of strain to form biofilms on stainless steel coupons at 37 °C and may enhance the expression of a clumping protein CluA, a significant adhesion factor of *Lactococcus* [14]. Therefore, despite that the *Lc. lactis* is hydrophilic, the bio adhesion was established on hydrophobic silicone. Indeed, according to our recent finding [8], silicone surface is hydrophobic and lowly electron donor and *B. cereus* surface is hydrophilic and electron donor. Indeed, we have showed that the adhesion of *B. cereus* despite the difference of hydrophobicity on silicone has modified the chemistry of surface which became hydrophilic and electron donor [8]. Hence, a decreasing of hydrophobic character of silicone surface with an increasing of electron donors character was noted could be correlated with *B. cereus* bio adhesion and metabolic activity on silicone [8]. We have hypothesized that the variation of chemistry of silicone favored the adhesion of hydrophilic *Lc. lactis* on the silicone surface and the production of biofilms may have influenced the final population of *B. cereus* on the surface, associated to competitive inhibition. In fact, we have hypothesized that

Fig. 2 *Bacillus cereus* biofilm formation on silicone flow system at 3, 24 and 48 h. **a** Electro micrographs by ESEM. **b** Multiparametric flow cytometry dot plots

Table 1 Assessment of the impact of *Lc. lactis* bio adhesion on *B. cereus* biofilm

	Biofilm formation (log ₁₀ cfu/cm ²)			Impact of bio adhesion (log ₁₀ cfu/cm ²)		
	3 h	24 h	48 h	3 h	24 h	48 h
<i>B. cereus</i>	6.92 (3.4)	9.16 (4.2)	11.5 (1.2)	7.21 (1.4)	4.34 (2.9)	2.12 (4.3)
<i>Lc. lactis</i>				8.15 (3.0)	9.19 (5.1)	9.14 (2.4)

( ): Standard deviation

Lc. lactis bio adhesion can be induced the insufficiency of oxygen on the silicone interface with adhered cells, and consequently it was able to remove *B. cereus*

biofilm. Indeed, Mahdavi et al. [20] reported that air–liquid interface appears to be very important in enhancing the attachment of bacteria to surface, and biofilm development

Fig. 3 Impact of bio adhesion of *Lc. lactis* on silicone adhered by *B. cereus* biofilm at 3, 24 and 48 h. **a** Electro micrographs by ESEM. **b** Multiparametric flow cytometry dot plots

Table 2 Contact angles values, free energy of interaction (G_{wi}) and surface energy and their tension components of *Lc. lactis*

	Contact angles (°)			Surface tension: components and parameters (mJ m ⁻²)					G _{wi}
	θ _w (°)	θ _F (°)	θ _D (°)	γ ^{LW}	γ ⁺	γ ⁻	γ ^{AB}	γ ^{tot}	
<i>Lc. lactis</i>	12.6 (1.37)	14.8 (0.85)	43.8 (2.9)	37.6	1.5	54.1	18.01	55.6	3.95

( ): Standard deviation

θ_w (°) water contact angle, θ_F (°) formamide contact angle, θ_D (°) diiodomethane contact angle, γ^{LW} Lifshitz van der Waals compound, γ⁺ electron acceptor compound, γ⁻ electron donor compound, γ^{AB} acid–base compound, γ^{Tot} total energy γ^{AB} + γ^{LW}, G_{wi} total free energy of interaction

was found to be sensitive to oxygen and nutrients availability. Also, these findings were consistent with Habimana et al. [14] that highlighted that multiplication of the

undesirable organism may be inhibited by nutrient competition or by the synthesis of antagonistic compounds such as acids, bacteriocins, polysaccharides or surfactants,

which demonstrate good anti-adhesive properties and antimicrobial activity.

Conclusion

This study has yielded that *B. cereus* readily formed biofilm on silicone surface under dynamic flow and static models deeply affects their structure and the biofilm biovolume and also we demonstrated that *Lc. lactis* bioadhesion may be a step in developing strategies to prevent microbial colonization of silicone-rubber. Indeed, this study revealed that formation of protective biofilm on industry workshop surfaces such as dairy and food industry, can be beneficial because their presence may effectively modify the physicochemical properties of substrates and favours the antimicrobial coating of devices make it simultaneously anti-adhesive and give it antimicrobial activity. So, this approach can constitute a natural and promising way of biofilm removal from industrial surface.

Acknowledgments We thank Mrs. Faten Nefzi for helping with the FCM analysis. Unité de Cytométrie en Flux (LR99-ES27), Faculté de pharmacie, Monastir, Tunisia.

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