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Piyush Kumar Jha [a], Heni Dallagi [a], Elodie Richard [b], Maureen Deleplace [a], Thierry Benezech [a], Christine Faille [a,*]

[a] Univ. Lille, CNRS, INRAE, ENSCL, UMET, F-59650, Villeneuve d'Ascq, France
[b] Univ. Lille, CNRS, INSERM, CHU Lille, Institut Pasteur de Lille, US 41 - UMS 2014 - PLBS, F-59000, Lille, France

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ABSTRACT

In the food industry, the control of surface hygiene is a major issue. It is therefore essential to identify those parameters that can affect the bacterial contamination of surfaces and the effectiveness of hygiene procedures. Numerous studies have focused on the role of bacteria, flow arrangements or materials, but almost nothing has been reported on the possible impact of vertical or horizontal positioning of surfaces on bacterial contamination. The aim of the current study was firstly to determine the ability of bacterial species usually found in food processing lines to form biofilms on surfaces positioned vertically or horizontally and then to assess the resistance of these biofilms to detachment. The experiments were carried out using three bacterial strains (*Escherichia coli* SS2, *Bacillus cereus* 98/4, and *Pseudomonas fluorescens* Pf1) that produced biofilms on glass, polypropylene and stainless steel (surface finishes 2B and 2R). We first observed that not only did the bacterial strain type impacts its ability to form biofilms (Ec-SS2 > Pf1 > Bc-98/4), but that the vertical vs horizontal position of the surface would also affect biofilm formation, probably due to the accumulation through sedimentation of bacteria on horizontal surfaces. However, the horizontally formed Pf1 biofilms were very fragile and could be partially removed by a gentle rinsing step. Lastly, no significant differences could be found in the ability to form biofilms on the different materials. The resistance to detachment to a standard rinsing process in a pilot rig was also investigated. While both strains and materials significantly affected the amount of biofilm detached, only Bc-98/4 biofilms were impacted by the surface position, with horizontal biofilms showing extreme resistance to shear forces. In conclusion, this study shows that horizontal surfaces in food environments probably represent an increased risk of contamination by bacteria frequently isolated from these environments and should be subjected to increased monitoring.

1. Introduction

Biofilms are often defined as assemblages of surface-associated microbial cells encased in a matrix made of self-produced extra-cellular polymeric substance (EPS). In the food industry, biofilm formation can occur on any surface, whether food contact surfaces including processing lines, slicing machines, washing tanks, cutting boards, or non-food contact surfaces such as walls, floors and drains. They therefore represent a serious threat to both food quality and food safety, especially as the biofilms are often strongly resistant to the cleaning (Faille et al., 2018) and disinfection (Araujo et al., 2011) procedures implemented to control surface contamination. Moreover, all types of materials are affected by contamination, including stainless steel, polymers and even glass. Once biofilms are established on a production or packaging line, it is therefore likely that there will be recurrent problems of cross-contamination of food, causing not only reduction of the shelf life of products (Galié et al., 2018) but also potential outbreak of foodborne diseases (Mazaheri et al., 2021).

It is often accepted that many factors affect the formation of biofilms and their properties, e.g. their structure or their resistance to rinsing and/or cleaning procedures. These include bacteria (Faille et al., 2014), flow arrangements (Bouvier et al., 2021), material's surface properties (Faille et al., 2018), or position of the surface within the processing line such as in fully submerged areas vs. at an air-liquid-wall interface (Jha et al., 2020).

Among these factors, the materials of contact surfaces have been the

* Corresponding author.
E-mail address: christine.faille@inrae.fr (C. Faille).

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subject of numerous investigations, in particular regarding their hydrophilic/hydrophobic characteristics and topography. However, many conflicting results have been reported on the role of the material on biofilm formation and its properties (Faille et al., 2018). These authors suggested that the hydrophilic/hydrophobic characteristics evolve over time, due in particular to surface conditioning by molecules with which the material comes into contact. This evolution could, at least partially, explain the difficulty in identifying one or more of the parameters related to the hygienic characteristics of the materials. The difficulty in assigning a specific role to each of the physico-chemical interactions could also be related to the coupling of several interactions in bacterial adhesion and biofilm formation (Sheng et al., 2007). As for the topography, it seems obvious that this would affect surface hygiene in any environment, if only because surface irregularities (peaks, crevices, etc.) would provide shelter for adherent bacteria from the shear stresses generated by flows during production or hygiene procedures. However, it is still difficult to identify a roughness parameter linked to surface hygiene, even if maximum values for parameters such as average roughness (Ra) or maximum roughness (Rz) have been proposed in normative or recommendation documents to define so-called hygienic materials. For example, the American 3-A Sanitary organization and the European Hygienic Engineering and Design Group (EHEDG) specify that food contact surfaces should have a maximum average roughness of 0.8 μm (which corresponds to a No. 4 finish on stainless steel).

Conversely, few studies have reported the role of the position or orientation of the surface in biofilm formation. However, previous studies conducted on pilot-scale washing tanks (used in the fresh-cut vegetable industry) have revealed that the surface position in different areas of the tanks impacted the amount and structure of *Pseudomonas fluorescens* and *P. grimontii* biofilms and their resistance to detachment (Cunault et al., 2018, 2019). For example, pronounced differences were observed between fully-immersed vertical and horizontal areas and were mostly explained by the flow arrangements (and thus the wall shear forces) within the tanks. However, for surfaces contaminated under static conditions, the biofilm formation by both *Pseudomonas* species were slightly higher on horizontal surfaces than on vertical surfaces although the differences were not significant. Consequently, the probable impact of positioning the surfaces vertically or horizontally on the installation of biofilms and their properties requires further studies.

The current study was designed to determine the ability of bacteria usually found on food processing surfaces to form biofilms on surfaces positioned vertically or horizontally. The impact of vertical/horizontal positioning and of material surface properties on the formation of biofilms (amount and morphology) and their resistance to detachment to a standard rinsing process in a pilot rig was investigated.

2. Materials and methods

2.1. Bacterial strains and materials

Three bacterial strains were used throughout this study. *E. coli* SS2 (subsequently named Ec-SS2) is a model pathogen expressing a green fluorescent protein (GFP) (Gomes et al., 2017), *P. fluorescens* Pf1 (Pf1) was isolated by ANSES from cleaning-in-place effluents (Cunault et al., 2015) and *B. cereus* CUETM 98/4 (Bc-98/4) was isolated from a dairy processing line (Lequette et al., 2010). Four materials were used in the form of 45 mm × 15 mm rectangular coupons to investigate the biofilm properties. Glass (Glasatelier Aillart, Meerhout, Belgium) was chosen because of its highly hydrophilic nature. The other three materials are found in food environments: AISI 316 stainless steel with pickled (2B) and bright annealed (2R) finishes (kindly provided by APERAM, Isbergues, France), and polypropylene (PP) (API Plastiques, Brenelles, France). Prior to each experiment, the coupons were cleaned and disinfected according to a previously described procedure (Jha et al., 2020). Briefly, coupons were cleaned using an alkaline detergent RBS T105 (Traitements Chimiques des Surfaces, France). One day (24 h) prior to the experiments, they were disinfected by heat-treatment, either in a dry heat oven at 180 °C for 1 h (2B and 2R), or by autoclaving at 121 °C for 30 min (PP and glass).

The roughness parameters of the four materials were calculated from the topography profiles obtained with a Perthometer S2 profilometer (Mahr, France). Ra is the arithmetical mean deviation of the absolute ordinate values within a sampling length and Rz is the sum of the height of the largest profile peak height and the largest profile valley depth, within a sampling length. The hydrophilic/hydrophobic characteristics were estimated by measuring the water contact angle with a DSA 100S goniometer (Kruss, Hamburg, Germany) of 1 μl droplets deposited at the material surface (5 droplets on 2 coupons for a total of 10 droplets). A material was considered hydrophobic for water contact angles over 90°, hydrophilic for lower values.

2.2. Biofilm production and resistance to a rinsing procedure

The cleaned and sterilized coupons were immersed vertically or horizontally in different soiling suspensions. For each strain, the conditions of biofilm formation were defined in such a way as to obtain sufficiently developed deposits (in horizontal and vertical positions) to allow their subsequent resistance to detachment. For the vertical soiling, whatever the strain, the coupons were fully immersed in polypropylene vials (3.4 cm × 7 cm) containing 50 ml of 1/10 TSB inoculated with 24 h-cultures of each strain (final concentration of around 10^7 CFU mL^{-1}). For horizontal soiling, coupons were placed in 14 cm diameter Petri dishes, covered with the same bacterial suspensions (1.2 cm above the plates for Ec-SS2 and Pf1; 2 mm for Bc-98/4). The preliminary tests showed that when the coupons were covered with 1.2 cm of suspension, the level of contamination of the surfaces by Bc 98/4 was low and that the residual amount after rinsing or cleaning was too small to allow any analysis. For both positions, the biofilms were left to form at 30 °C for 48 h for Ec-SS2 and Pf1, but only 24 h for Bc-98/4. For longer incubation times, a significant part of the Bc-98/4 biofilms seemed to have been released into the suspension, which made it difficult to analyze the influence of the different parameters. After incubation, the coupons were quickly rinsed with ultrapure sterilized water to remove any loosely bound cells, and then further analyzed (Section 2.3).

In order to determine the biofilm resistance to detachment, the rinsed coupons were first left to dry at room temperature for 1 h. In order to avoid possible inactivation of the biofilm bacteria by the adverse conditions encountered during a cleaning procedure due to the presence of NaOH, high temperature, a standard rinsing procedure was preferred to evaluate the resistance of the biofilms to detachment. The dried coupons were placed in rectangular test tubes, connected to a pilot rig and subjected to a rinsing procedure with water at 30 °C (20 min at 5 Pa). The residual biofilms were then analyzed as described below.

2.3. Biofilm analysis

To determine the bacterial counts on the surfaces, biofilms were collected using a dry cotton swab (Copan, Brescia, Italy). The swab was then placed in sterile swab tubes containing 2.5 ml of sterile saline solution and then vortexed for 1 min at a maximum speed of 2400 rpm. The detached bacteria present in the suspension were plated on TSA (Tryptone Soy Agar, Biokar, France), incubated at 30 °C for 48 h and then enumerated.

In order to study the biofilm microstructure, the coupons were dried at room temperature for 1 h, then stained with acridine orange (0.01%) for 15 min, rinsed gently with softened water and allowed to dry. Structural observation of biofilms was carried out by epifluorescence microscopy (Zeiss Axioskop 2 Plus, Oberkochen, Germany, x50 magnification). In addition, further observations were performed using a confocal laser scanning microscope (CSLM, Zeiss, LSM780, Oberkochen, Germany) at the x400 magnification with a 40× oil objective NA 1.3, to detect a potential 3-D organization of the biofilms. These observations

mainly focused on the clusters or, in the absence of clusters, the most contaminated areas of the coupons. This technology enables 3D reconstructions of the biofilms by stacking multiple two-dimensional images (Z stacks) obtained at different depths in the samples.

2.4. Statistical analysis

Each experiment was repeated three or four times. A series of statistical analyses were performed using SAS V9.4 software (SAS Institute, Gary, NC, USA). Variance analyses and Tukey's grouping (Alpha level = 0.05) were performed to determine 1) the influence of the bacterial strains on the amounts of biofilm (CFU); 2) for each strain, the respective role of materials and coupon positions on the biofilm amount (CFU); 3) the decrease in the CFU number following the rinsing procedure; 4) the influence of the bacterial strains on the decrease in the CFU number (in terms of log reduction); 5) for each strain, the respective role of materials and coupon positions during biofilm formation on the decrease in the CFU number; and 6) the influence of the bacterial strains on the amount of residual biofilm (in terms of CFU).

3. Results

3.1. Material surface properties

First, as shown by the roughness values obtained by profilometry (Table 1), topography differed considerably according to the materials, which was confirmed by the variance analysis ($p < 0.0001$). Stainless steel 2B was clearly the roughest. The high Rz value (1.52 μm) may reflect not only the presence of the grain boundaries, but also that of surface defects. Differences could also be observed among the other materials. 2R stainless steel and polypropylene were relatively smooth and belonged to the same class according to Tukey's grouping, while glass was significantly smoother.

The material's hydrophobicity was estimated through the measurement of the water contact angles. The greater the contact angle, the more hydrophobic the material was. As in the case of roughness, the materials were significantly different in terms of hydrophobicity ($p < 0.0001$). As shown in Table 1, glass was strongly hydrophilic, while polypropylene exhibited a marked hydrophobic character. 2R and 2B stainless steels gave intermediate values. The Tukey's analysis indicated that the materials are all significantly different and ranked from the most hydrophobic to the most hydrophilic: PP > 2B > 2R > glass.

3.2. Enumeration of the biofilms formed on vertical vs horizontal surfaces

Data obtained by enumeration of cultivable cells within the biofilms are presented in Fig. 1A. First, the number of cultivable cells was clearly dependent on the strain. Indeed, whatever the material and the position, the surface contamination ranged from 4.20 10^6 to 2.16 10^7 CFU cm^{-2} for Ec-SS2, followed by Pf1 with between 7.35 10^5 and 3.95 10^6 CFU cm^{-2}. Concerning Bc 98/4, even under the specific conditions defined to promote biofilm formation, this strain produced at best 2.57 10^5 CFU cm^{-2} in the different conditions tested. The amount of biofilm was also affected by the vertical or horizontal position of the coupons, but to a lesser extent. More interestingly, the influence of the position differed between the three bacterial strains. Ec-SS2 and Bc-98/4 biofilms were produced in lower quantities when the coupons were placed vertically, whereas the opposite was observed for Pf1. It is noteworthy that preliminary tests had shown that Bc-98/4 produced very small amounts of biofilm on horizontal surfaces placed at a depth of 1.2 cm (as used for the other two strains, Ec-SS2 and Pf1). The depth of the horizontal surface therefore also appeared to play a significant role. Lastly, the type of material seemed to have little or no effect on biofilm formation, except perhaps for Bc-98/4 whose CFU counts were higher for biofilms formed on PP, especially in the vertical position.

Variance analysis taking into account all the data, showed that the three parameters (bacterial strain, material, and position) accounted for 85% of the variability and confirmed the significant role of the bacteria on the amount of cultivable cells ($p < 0.0001$). The Tukey's grouping showed that the three strains belonged to different groups (A, B, and C for Ec-SS2, Pf1 and Bc-98/4, respectively). Further statistical analyses were performed for each strain in order to investigate the respective role of the materials and the vertical/horizontal positions. Whatever the strain, the material had no significant effect on the biofilm formation, unlike the position ($p < 0.0001$ for the three strains).

Table 1
Characterization of the surface properties of the four materials. The hydrophilic/hydrophobic character was estimated by the water contact angle measured by goniometry. The roughness parameters were measured using a profilometer.

	2R	2B	PP	Glass
θ water				
Average value	64.0	76.0	99.7	17.0
Tukey's grouping[a]	C	B	A	D
Average roughness (Ra)				
Average value (μm)	0.03	0.21	0.03	0.00
Tukey's grouping[a]	B	A	B	C
Maximum roughness (Rz)				
Average value (μm)	0.23	1.52	0.26	0.02
Tukey's grouping[a]	B	A	B	C

[a] Tukey's grouping (groups with common letters are not significantly different).

3.3. Structures of the vertically and horizontally-formed biofilms

In order to investigate the distribution of the biofilms on the surface of the materials, the coupons were first observed by epifluorescence microscopy at x50 magnification (Fig. 2). The surface coverage levels were consistent with the enumeration results, which were lowest for the Bc-98/4 biofilms, and highest for the Ec-SS2 biofilms. The differences noted above pertaining to the role of the coupon position are globally confirmed by microscopy in that the Bc-98/4 biofilms were denser in the horizontal position, but the opposite was observed for Pf1. On the other hand, it was difficult to draw conclusions from the observations of the Ec-SS2 biofilms, perhaps due to the high contamination levels of the different coupon, or to their complexity.

Differences also occurred in the organization of the different biofilms. First, for Ec-SS2, numerous cell clusters were observed on all the vertical and horizontal coupons, some reaching a width of a few tens of μm, even 100 μm. A network structure was often observed between these clusters. Interestingly, bubbles were repeatedly observed during biofilm formation by Ec-SS2, on the surface of PP coupons whether placed vertically or horizontally yet less often on the surface of 2B coupons. The presence of these bubbles was revealed on the biofilms in the form of rounded areas with little contamination except in one area where the biofilm seems to have concentrated (Fig. 2, Ec-SS2 on PP vertical, Fig. 3, Ec-SS2 on 2B horizontal). Conversely, Bc-98/4 and Pf1 biofilm structures were clearly affected by the vertical or horizontal position of the coupons. Irrespective of the material, the Bc-98/4 biofilms were constituted of more or less isolated cells or of small cell-clusters, separated by areas free of any contamination, although these were more extended in the case of vertically-placed coupons. Pf1 biofilm structures were even more markedly affected by the position of the coupons. Indeed, while numerous and sometimes large clusters separated by less contaminated areas were observed on the vertical coupons, the biofilms were only organized in a network structure with few cell clusters on the horizontal ones.

We then investigated the presence of spores within the biofilms of Bc-98/4 produced horizontally. The use of a x1000 magnification was required here in order to facilitate observation after staining biofilms with acridine orange. Under these conditions, the spores were yellow or

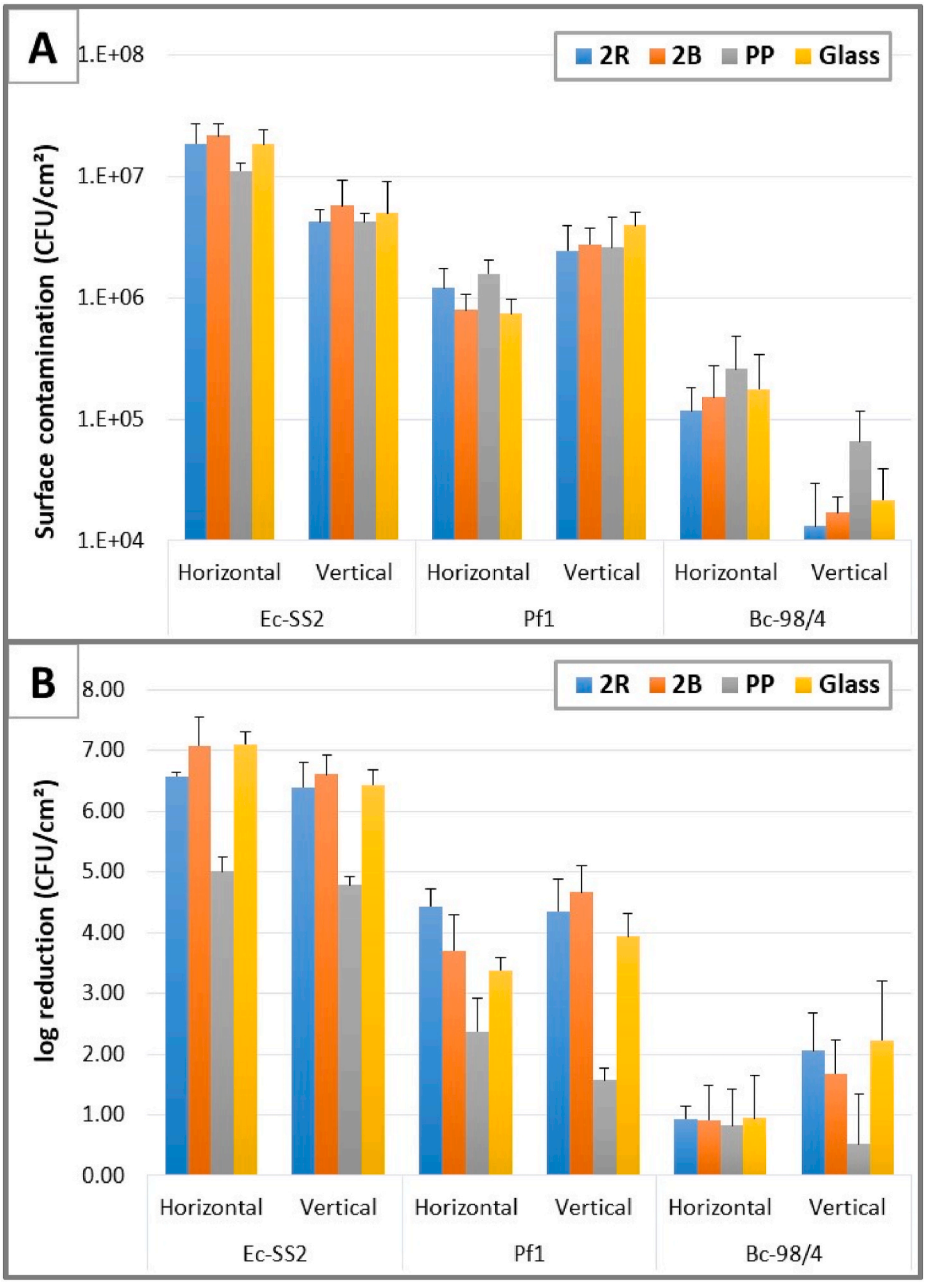

Fig. 1. (A) Bacterial load (CFU) of the three strain on different materials placed vertically and horizontally. (B) Log reduction of the number of CFU induced by standard rinsing procedure (20 min at 5 Pa and at ± 30 °C).

green and the vegetative cells orange. As shown in Fig. 4, many spores were embedded in the Bc-98/4 biofilm clusters. Furthermore, in some clusters (e.g. for biofilm produced on 2R and glass), most cells appeared to be sporulating.

In order to observe the structure of the clusters or the most contaminated areas of the coupons in more detail, further observations were carried out by CSLM at a x400 magnification (Fig. 3). Despite their size, even the large clusters formed by Ec-SS2 were relatively flat, whether positioned vertically or horizontally. These observations also highlighted the network arrangement between the clusters, except perhaps on the glass. Moreover, even for vertical surfaces poorly contaminated with Bc-98/4, the presence of small cell clusters as well as of chains of cells demonstrates that the adherent cells were indeed multiplying and, in other words that we are dealing with biofilm formation and not with simple bacterial adhesion.

In summary, despite clear differences in contamination rate and structure, all biofilms were thin (probably no more than one or two cell layers) and were composed of clusters and single or chains of cells.

3.4. Enumeration of the residual biofilms after a rinsing procedure

Biofilms were then subjected to a rinsing procedure at a wall shear stress of 5 Pa. The reduction in the number of cultivable cells following the rinsing procedure is presented in Fig. 1B (log reduction). Depending on the strain, material and position, huge differences were observed, with the log reduction of the CFU number ranging from 0.5 (Bc-98/4 on PP in the vertical position) to 7.1 (Ec-SS2 on glass and 2B in the horizontal position). Regarding the impact of the individual strains studied, Bc-98/4 was the most resistant to rinsing and Ec-SS2 the least resistant, whatever the experimental conditions. Analysis of the impact of the materials showed that the decrease in the CFU number was the lowest on the hydrophobic PP, whereas no trend could be detected for the other

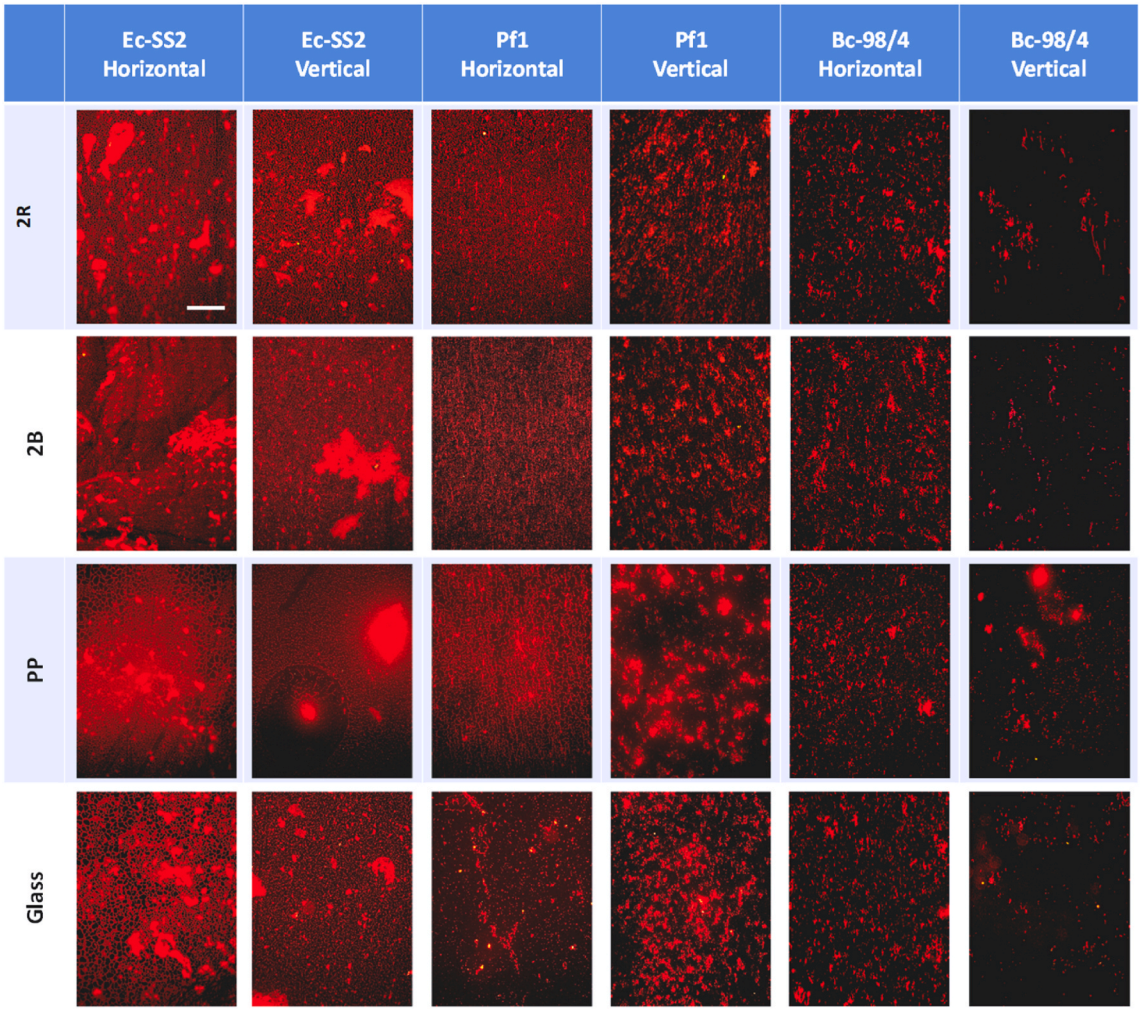

Fig. 2. Microscopic images of biofilms grown on different materials in horizontal and vertical positions and stained with orange acridine. White bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

materials. However, there was an exception for Bc-98/4 biofilms produced on horizontal coupons, which demonstrated almost no detachment, even on PP. Finally, it was difficult to draw conclusions concerning the role of the position by observing Fig. 1B alone, except for Bc-98/4, whose horizontally-formed biofilms were extremely resistant to the shear stresses generated by the water flow, whatever the material.

Statistical analysis confirmed the efficiency of the rinsing procedure ($p < 0.0001$), even on Bc-98/4. When log reductions of the three strains were considered, the statistical analysis indicated that the three parameters accounted for 94% of the variability observed on the log reduction and that the decrease in the number of CFU significantly differed between strains ($p < 0.0001$), each strain belonging to a different Tukey group. It was of particular interest to note that even though the material did not impact biofilm formation, it clearly affected the log reduction for all strains, although it had a lesser impact on Bc-98/4, mainly for horizontal biofilms, probably because of their extreme resistance to detachment ($p < 0.001$ on Ec-SS2 and Pf1, $p = 0.0055$ on Bc-98/4). Regarding the impact of the coupon position, no clear trend emerged from this study since the decrease in the CFU number of vertical biofilms was greater than those of horizontal biofilms for Bc-98/4, slightly lower for Ec-SS2, and finally similar for Pf1.

Considering the levels of residual contamination after rinsing, it first appeared that the CFU number was lowest for vertically and horizontally-formed Ec-SS2 biofilms and highest for horizontally formed Bc-98/4 biofilms. Furthermore, PP remained more contaminated than the other materials in all conditions tested except for horizontally-formed Bc-98/4 biofilms, as there was no influence of the materials in these conditions.

3.5. *Structure of the residual biofilms after the rinsing procedure*

The rinsed coupons were observed by epifluorescence microscopy at x50 magnification (Fig. 5). The most striking observation was that the rinsing procedure hardly affected either surface coverage or biofilm structure, as would be expected from the enumeration results. Indeed, cell clusters, sometimes even very large ones as well as network structures were still present. In contrast, far fewer isolated cells were observed on the different biofilms subjected to the rinsing procedure. The contamination level seemed to be lower after rinsing, which suggests that the biofilms had been partially removed. It is therefore very likely that most of the cells still present on the surfaces after rinsing were no longer cultivable, especially for the Sc-SS2 strain. Finally, these observations do not allow a conclusion to be drawn as to a possible influence of the materials and/or the positions of the coupon on the resistance of biofilms to rinsing.

4. Discussion

In the food industry, equipment surfaces may be horizontal, vertical, or in any position in between, either on open surfaces or of course in pipes and complex equipment (e.g. pumps, valves). However, little attention has been paid to the impact that surface position can have on

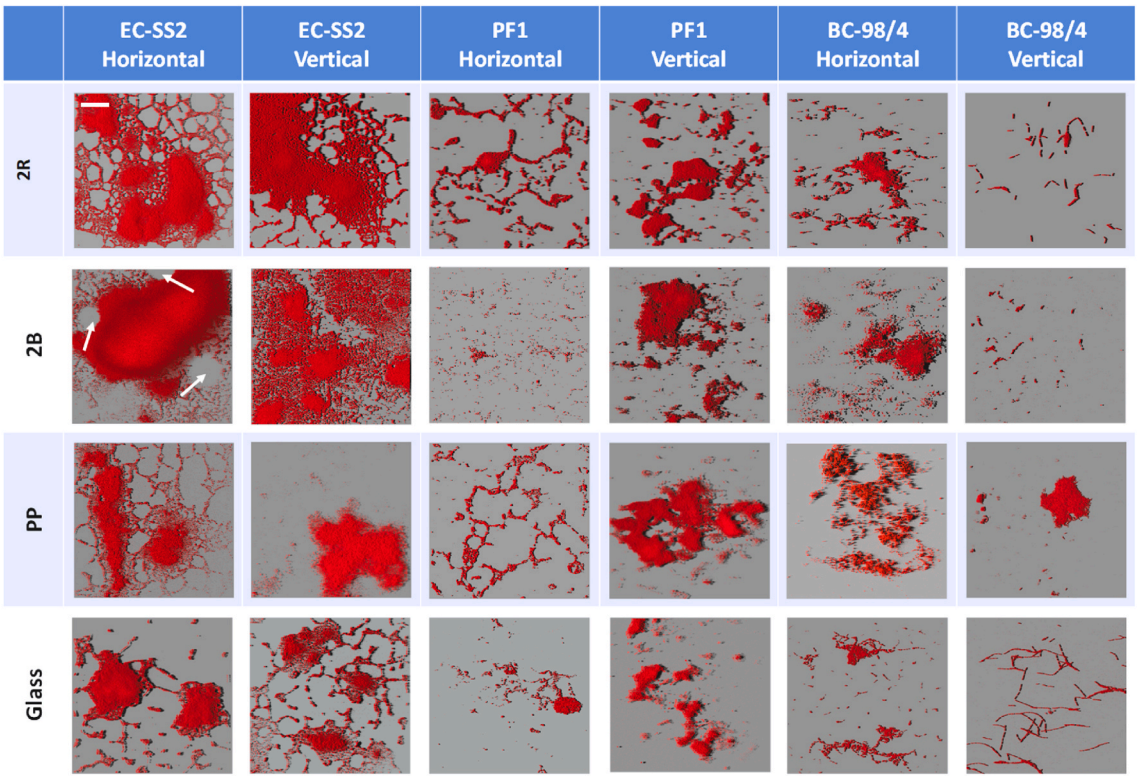

Fig. 3. Biofilms formed on different materials in vertical and horizontal positions, examined with a confocal scanning laser microscope. White arrows = marks probably left by micro-bubbles. White bar = 50 μm.

the formation of biofilms and on their further resistance to cleaning.

In this study, we first explored the ease of bacteria in forming submerged biofilms on materials with different surface properties, placed in a vertical or horizontal position. In order to avoid any interaction with more or less complex flow arrangements, the biofilms were produced under static conditions. The three bacterial species tested are known to be able to form biofilms and have often been isolated from food environments. For example, many bacteria belonging to the *E. coli* species can adhere to various materials (Galié et al., 2018) including stainless steel, glass, and polymers (e.g. Teflon and polypropylene) and many *E. coli* strains isolated from food have shown a strong propensity to produce biofilms (Badi et al., 2020). Similarly, the majority of *P. fluorescens* strains isolated from dairy manufacturing plants has been shown to be able to form biofilms (Rossi et al., 2016). Furthermore, strains belonging to this species can contaminate different materials (Wan Dagang et al., 2016) and the interaction forces between biofilms and materials are known to be weaker on glass than on stainless steel. Lastly, *B. cereus* is responsible for biofilm formation on surfaces of food and beverage industries and has been isolated from surfaces of closed and open equipment such as conveyor belts, stainless steel pipes, conveyor belts and storage tanks (Majed et al., 2016). Biofilms were produced on four materials characterized by different surface properties. Glass is very hydrophilic and very smooth, polypropylene is hydrophobic and relatively smooth and the two types of stainless steels tested have intermediate properties, with the 2R surface finish being slightly smoother and more hydrophilic than the 2B surface finish. However, as the roughest material is characterized by an average roughness Ra of 0.21 μm (2B), which is well below the often-recommended threshold value of 0.8 μm (e.g. by the EHEDG and the 3-A organization), the 4 materials are considered to be hygienic.

As expected, the three strains tested have very different biofilm forming propensities on the different materials, both in the number of CFU and in the organization of the biofilm (mainly with regard to the size of the clusters). This phenomenon is widely known and has been reported in the literature for strains belonging to different genera or species (Cherif-Antar et al., 2016), or even to the same species such as *B. cereus* (Majed et al., 2016) or *P. fluorescens* and other *Pseudomonas* species (Ude et al., 2006).

Whatever the bacterial strain and despite the large differences in the surface properties (topography, physicochemistry), the material failed to significantly affect either the biofilm quantity or structure, whether the surfaces were placed vertically or horizontally. This result was not very surprising, especially since experiments were performed in static conditions and none of the materials tested were assumed to have an inhibiting effect on bacterial growth. However, it should be noted that some works reported in the literature have suggested a role of hydrophobicity on biofilm formation, although these results are often inconsistent. For example, strains of *L. monocytogenes* (Bonsaglia et al., 2014) and *Staphylococcus aureus* (Lee et al., 2015) were reported to form larger amounts of biofilms on hydrophilic materials such as stainless steel and glass, than on polystyrene which is hydrophobic. Conversely, one day-old *Cronobacter sakazakii* biofilms were denser when produced on hydrophobic silicone or polycarbonate surfaces than on stainless steel (Jo et al., 2010). However, other authors failed to demonstrate any significant differences between the amounts of *S. aureus* biofilms formed on hydrophilic stainless steel and hydrophobic materials, such as polystyrene (da Silva Meira et al., 2012) or polyurethane and polyethylene (Abeysundara et al., 2017). Contradictory results have also often been reported for roughness, such as whether average roughness does affect (De-la-Pinta et al., 2019) or does not affect (Wu et al., 2018) biofilm formation.

Regarding the influence of the vertical or horizontal positioning of the surfaces, intuitively, one would expect greater amounts of biomass on horizontal surfaces due to the sedimentation of cells in the fouling suspension. In fact, a slightly higher level of contamination of horizontal surfaces by Ec-SS2 and Bc-98/4 strains was indeed observed, yet the opposite was found for the Pf1 strain. One possible explanation for the relatively small amount of Pf1 biofilms on horizontal surfaces is that

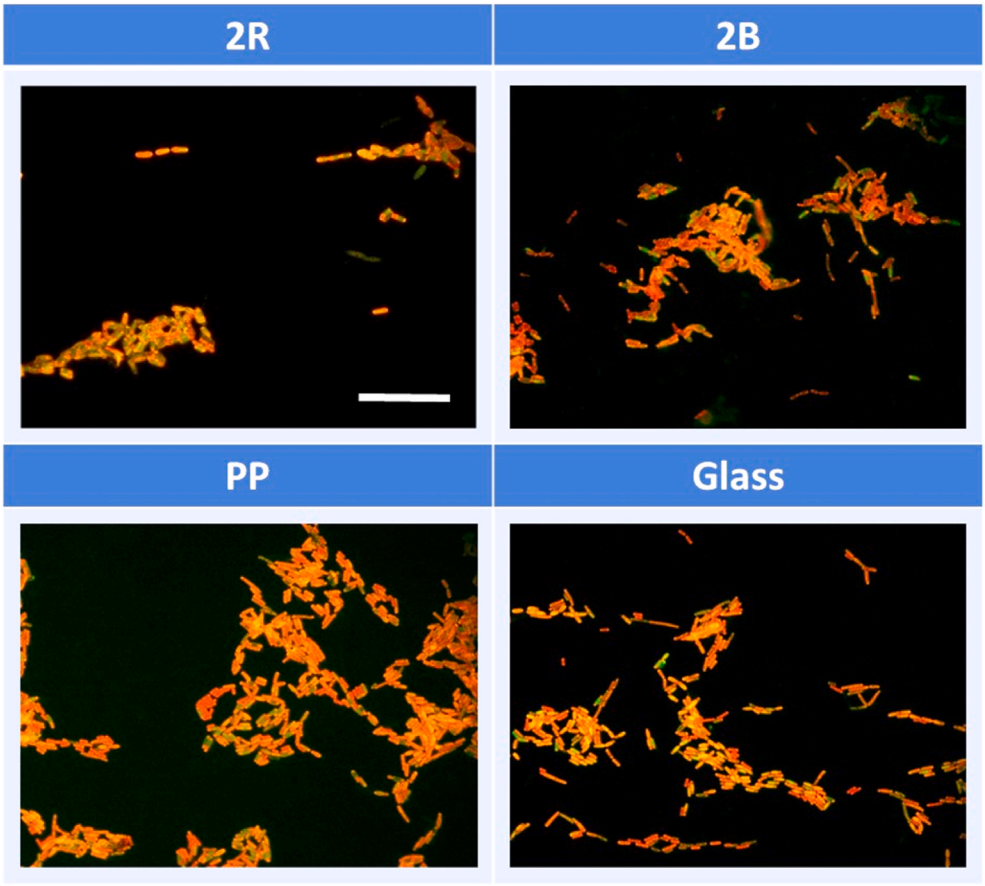

Fig. 4. Observation of spores within *B. cereus* biofilms formed horizontally on different materials. Images obtained by epifluorescence microscopy at a x1000 magnification. Spores are coloured green, vegetative cells orange. White bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

large amounts of biofilm would be produced by Pf1, but that this biofilm would be very fragile and easily removed. Indeed, the gentle rinsing procedure carried out before the analysis of the biofilm properties removed a significant part of this biofilm (Supplementary Figure), a phenomenon which was not observed for any other biofilm. The only other works carried out on the influence of vertical or horizontal surface positioning on biofilm formation concerned *Pseudomonas* biofilms produced under almost static conditions, i.e. at a wall shear stress of less than 0.01 Pa (Cunault et al., 2018, 2019). Under these conditions, the amount of biofilm was slightly greater on horizontal surfaces, although the differences were insignificant. This would suggest that sedimentation only plays a minor role in biofilm formation on horizontal surfaces. Other researchers have investigated the possible role of sedimentation on biofilm formation and their results have so far been inconclusive. For example, researchers involved in the investigation of the role of motility on biofilm formation by a strain of *B. cereus*, have suggested that sedimentation of non-motile bacteria would indeed promote biofilm formation on the bottom glass slide of a flow cell (Houry et al., 2010). Yet, in another study conducted on *E. coli* strains with different sedimentation propensities, biofilm formation was not found to correlate clearly with the sedimentation rate (Kessler et al., 2021). Finally, differences sometimes appear in the structure of the biofilms produced on vertical and horizontal surfaces, but no general trend was observed. It also seems likely that, in addition to sedimentation, other factors play a determining role in the formation of biofilms under different conditions. This would be particularly true for some intrinsic properties of the bacteria, such as their mobility (Zheng et al., 2021) and more particularly their aerobic/anaerobic character (Chang et al., 2015), which could at least partially mask the influence of the sedimentation phenomenon. However, our work suggests that when environmental conditions do not affect biofilm production (e.g. lack of oxygen), the sedimentation of planktonic bacteria would result in a more marked contamination of horizontal surfaces.

The biofilm resistance to a rinsing step was then investigated. Regardless of position and material, the decrease in the number of cultivable cells following the rinsing procedure was strongly marked for Ec-SS2 (over 4.8 log), very small for Bc-98/4 (below 2.2 log) and intermediate for Pf1. A possible reason for the high resistance of Bc-98/4 biofilms might be the presence of spores within the biofilms, as observed in a previous study of 48-h *B. cereus* biofilms (Faille et al., 2014). In contrast, the high sensitivity of *E. coli* biofilms has already been demonstrated in the laboratory. Indeed, a very gentle rinsing procedure (0.5 Pa) induced a decrease in the viable contamination of *E. coli* of almost 2.5 log while no significant decrease was observed for the other two species tested: *Klebsiella pneumoniae* and *Citrobacter freundii* (Faille et al., 2003). Despite the low CFU number obtained after rinsing the Ec-SS2 biofilms, large amounts of biofilms were still observed by microscopy, suggesting that the residual cells had lost their cultivability. Indeed, we were able to show that the 1 h drying step implemented to improve the reproducibility of the results, induced a loss of cultivability of the Ec-SS2 biofilms (2 log reduction on 2R), but not of the two other strains. Thus, if we assume that all Ec-SS2 biofilms had undergone a similar level of inactivation, i.e. close to 99%, their actual detachment level would be similar to that of Pf1 biofilms.

Concerning the role of materials, larger amounts of residual cultivable cells were seen on most biofilms formed on PP than on other materials, in line with previous results (Faille et al., 2002). However, the observation of the surfaces by microscopy did not allow conclusions to

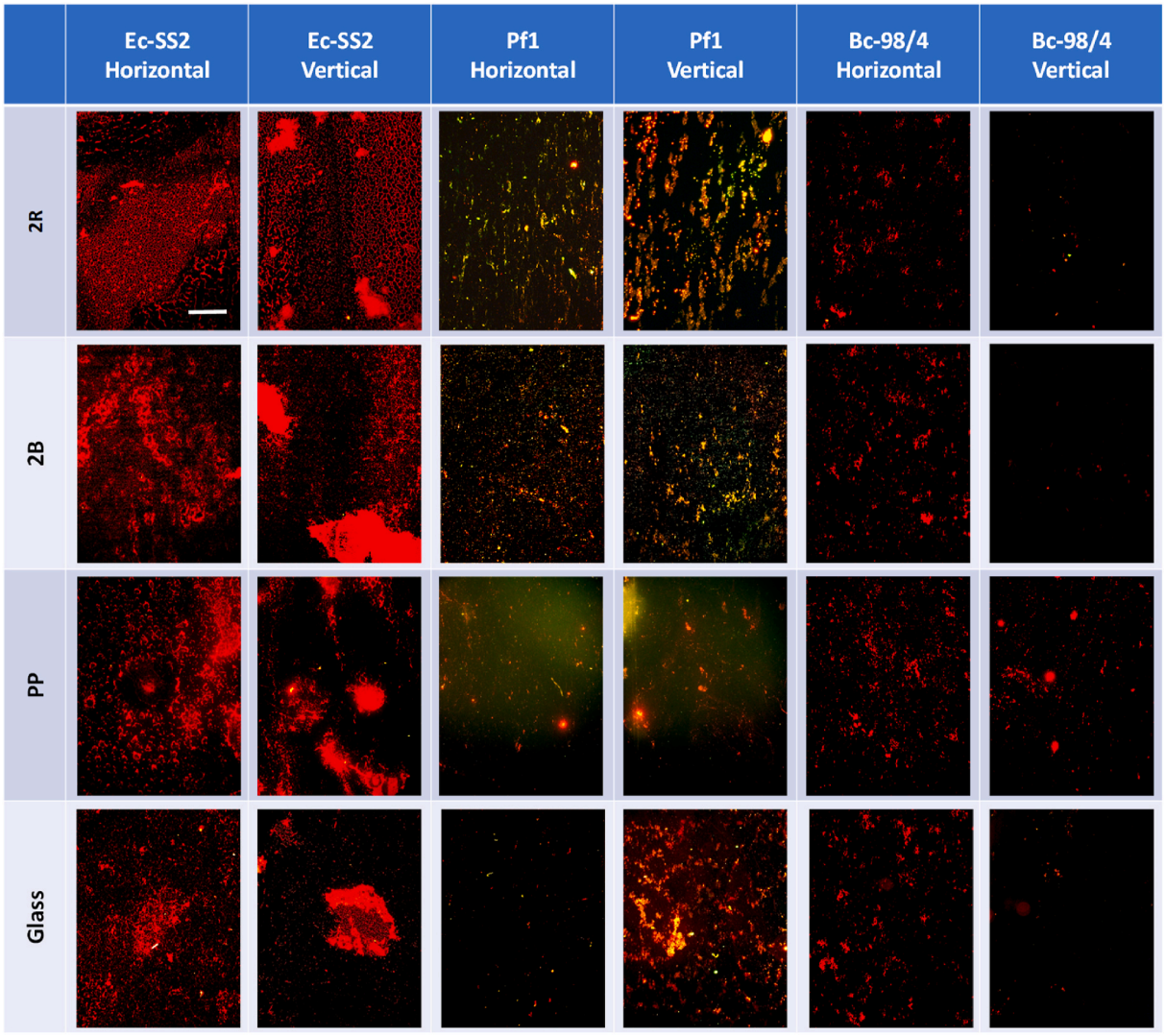

Fig. 5. Microscopic images of residual biofilms after a rinsing procedure (20 min at 5 Pa). White bar = 50 μm.

be drawn as to the amount of residual biomass on the different materials. In any case, one explanation of the differences between materials could be that the interaction forces would be stronger on a hydrophobic material than on a hydrophilic material. Indeed, the topography does not seem to play a role since the SS-2R and PP have similar roughness and the rougher SS-2B is less contaminated after the flushing procedure. However, where biofilm formation is concerned, contradictory results have been reported in the literature. Indeed, when adhesion forces were estimated by atomic force microscopy (AFM), *S. aureus* was shown to strongly adhered to 316L stainless steel, but very weakly to polyethylene (Alam & Balani, 2017), while *Enterococcus faecalis* exhibited great differences between polymers (Sénéchal et al., 2004), with detachment forces being higher on polyurethane (slightly hydrophilic) than on Teflon (strongly hydrophobic). Conversely, the adhesion force of *B. mycoides* spores (Bowen et al., 2002) was much lower on hydrophilic glass than on hydrophobic-coated glass and similar observations have also been made on interaction forces between *E. coli* on mica, hydrophilic glass, hydrophobic glass, polystyrene, and Teflon (Ong et al., 1999). An alternative hypothesis might be that the physiological state of the biofilm cells is impacted by the properties of the materials, as suggested by Sénéchal et al. (2004) for *E. faecalis* biofilms. Indeed, other authors have studied the membrane integrity of *L. monocytogenes* cells adhering to different materials including stainless steel, glass, and PP (Silva et al., 2008) and found that 100% of cells attached to the PP surface had an intact membrane, but this proportion felt to around 80% on glass and stainless steel. However, despite these differences in membrane integrity, cell viability was similar on glass and PP, lower on stainless steel, which is not consistent with the results reported here.

Finally, no general trend emerged from this study regarding the influence of the vertical or horizontal position on the ease of detachment of biofilms. The only remarkable difference is that Bc-98/4 biofilms were very resistant to detachment, especially when produced horizontally.

In conclusion, biofilms formed on horizontal surfaces were denser than those formed on vertical surfaces, but they were sometimes more fragile, and therefore failed to resist even very low shear forces. On the other hand, one of the three strains tested was more resistant to rinsing when formed in a horizontal position, whatever the material. Conversely, no differences were observed for the other two strains tested. Therefore, it is likely that in the food industry, these horizontal surfaces may pose a significant risk in terms of surface hygiene and should be subject to increased monitoring.

CRediT authorship contribution statement

Piyush Kumar Jha: Investigation, Methodology, Writing – original draft. **Heni Dallagi:** Investigation, Methodology. **Elodie Richard:** Investigation, Methodology. **Maureen Deleplace:** Investigation.

Thierry Benezech: Funding acquisition, Project administration. **Christine Faille:** Supervision, Conceptualization, Validation, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.108646.

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