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1 **Biofilm dispersal induced by mechanical cutting leads to heightened foodborne**
2 **pathogen dissemination**

3

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Abstract:

The biofilm life cycle where bacteria alternate between biofilm and planktonic lifestyles poses major implications in food spoilage and gastrointestinal infections. Recent studies had shown that freshly biofilm-dispersed cells have a unique physiology from planktonic cells, raising the fundamental question if biofilm-dispersed cells and planktonic cells disseminate differently across food surfaces. Mechanical dislodging via cutting can cause biofilm dispersal and eventual food cross-contamination. Here, we showed that biofilm-dispersed bacteria from various foodborne pathogens were transferred from freshly cut surface at a higher rate to the cutting material than that of planktonic bacteria. When the cutting tool was used to cut a fresh surface, more biofilm-dispersed bacteria were disseminated from the cutting tool to the newly cut surface than planktonic bacteria. Our observations were applicable to cutting tools of various materials and cut surfaces, where polystyrene and surfaces with high water content were most susceptible to biofilm transfer, respectively. Simple washing with detergent and mechanical wiping could aid bacterial removal from cutting tools. Our work revealed that biofilm-dispersed cells were transferred at a higher rate than planktonic cells and cutting tool was an important medium for pathogen cross-contamination, thus providing insights in maintaining their cleanliness in food processing industries.

Keywords: Biofilm; Planktonic bacteria; Dispersed cells; Mechanical dislodging

40 **Introduction:**

41

42 Bacteria can exist as planktonic free-swimming individual cells or mostly as biofilms
43 cells. Both planktonic and biofilm cells possess different physiologies (Chua et al.,
44 2014), which caused major problems in the elimination of foodborne pathogens on food.
45 The biofilm life cycle where biofilm and planktonic lifestyles alternate is mediated by the
46 c-di-GMP secondary messenger signalling system found in most bacterial species
47 (Hengge, 2009).

48

49 Many foodborne pathogens, such as *Staphylococcus aureus*, enteropathogenic
50 *Escherichia coli* (EPEC), *Salmonella enterica* and opportunistic *Pseudomonas*
51 *aeruginosa*, form biofilms on food, leading to contamination and spoilage (Galié et al.,
52 2018). There is significant impact on human health and food industry, where biofilms
53 are difficult to clear and can disseminate easily to new sites (Galié et al., 2018).
54 The use of cutting tools, such as knives and blenders, is common in kitchens and food
55 processing industries, where raw food such as salads and fruits are cut and packaged.
56 While a recent study had shown that cutting tools play a role in dissemination of
57 pathogens on food (Erickson et al., 2015), it is unclear if biofilm cells can disseminate
58 similarly as planktonic cells via cutting tools of different material and cutting material of
59 various textures. Understanding the differences in biofilm-dispersal and planktonic
60 lifestyles in the cross-contamination of foods will offer insights into prevention and
61 eradication of bacteria on food products, and maintenance of hygiene on the knives.

62

63 Here, as proof-of-concept, we showed that biofilm-dispersed cells from various
64 foodborne pathogens could be transferred to the fresh agar surface from the
65 contaminated agar surface more effectively than planktonic cells, via mechanical
66 dislodging with a cutting tool. Using *P. aeruginosa* as model foodborne organism of
67 biofilm formation, we found that c-di-GMP signaling-controlled exopolysaccharides
68 played a crucial role in biofilm attachment to the cutting tool. Biofilm-dispersed cells
69 could be disseminated regardless of cutting tool material, such as stainless steel,
70 polystyrene plastic and ceramic were used, though plastic was the most susceptible to
71 biofilm attachment and dissemination. However, when using different concentrations of
72 agar which reflect the controlled environment and potato slices which reflect the realistic
73 and complex scenarios, we found that biofilm-dispersed cells are preferentially
74 transferred on moist soft surfaces (0.375% agar and cooked potato slices), indicating
75 certain food surfaces play a role in biofilm transfer. We showed that a combination of
76 detergent treatment and mechanical wiping could remove biofilms from the cutting tool,
77 indicating the necessity of proper disinfection of cutting tools in the food industry.
78

79 **Results:**

80

81 **Biofilm-dispersed cells are easily transferred to fresh media by cutting than**
82 **planktonic bacteria**.

83

84 Using *P. aeruginosa* as proof-of-concept, we first established a model to compare the
85 ability of planktonic bacteria and biofilm-dispersed cells to disseminate from the
86 contaminated surface to fresh surface via the cutting tool, where we used a ceramic
87 knife to cut the original matrix (1.5% agar or food) contaminated with similar starting
88 concentrations of planktonic and biofilm bacteria (**Supplementary Figure 1a-1c**),
89 followed by a subsequent cut on the fresh matrix (**Figure 1a**). We cut the agar piece for
90 quantification of bacterial numbers on the original matrix, fresh matrix, and knife and
91 tabulated the rate of transfer. We found that biofilm bacteria were dispersed from
92 original agar to knife (**Figure 1b; Supplementary Figure 1c**) and from knife to fresh
93 agar (**Figure 1c; Supplementary Figure 1c**) at a higher ratio than planktonic cells. Our
94 findings were corroborated with confocal laser scanning microscopy (CLSM) by
95 visualizing the localisation of *P. aeruginosa* tagged with constitutively-expressed
96 fluorescent *gfp* gene on the three surfaces (**Figure 1d**). We also employed a previously
97 established biofilm biosensor, p_{cdrA}-*gfp* (Chua et al., 2016), in the *P. aeruginosa*, where
98 we showed that biofilm cells on agar had high GFP expression of p_{cdrA}-*gfp* biosensor,
99 while planktonic cells on agar had no visible GFP expression of p_{cdrA}-*gfp* biosensor
100 (**Supplementary Figure 1d**), indicating that biofilm indeed had formed on the agar over
101 24 hrs.

102 By using other foodborne pathogens (*S. aureus*, *S. enterica* and *V. cholerae*)
103 (**Supplementary Figure 2**) and cutting tools made from other materials (polystyrene
104 plastic and stainless steel) (**Supplementary Figure 3**), we also found qualitatively
105 similar results where biofilm-dispersed cells disseminate more readily than planktonic
106 cells, indicating that our findings are applicable to other bacterial species and different
107 cutting material. As proof-of-concept, we continued to employ *P. aeruginosa* as our
108 choice of bacteria and ceramic knife as our choice material for downstream
109 experiments.
110
111 **Biofilm matrix is important to efficient biofilm transfer via cutting.**
112
113 We next determine which component of the biofilm matrix that plays a crucial role in
114 biofilm transfer during the cutting process. We tested our in-house mutant library of
115 biofilm matrix components (Chan et al., 2021), which included Δ*pelA*, Δ*pslBCD*,
116 Δ*pelA*Δ*pslBCD*, Δ*cdrA,* and Δ*pqsC*. *P. aeruginosa* produces Pel and Psl which are
117 exopolysaccharides, CdrA which is biofilm adhesion protein, and eDNA via *pqs* operon
118 (Mann and Wozniak, 2012). Loss of expolysaccharides resulted in poor transfer of
119 biofilms from original agar to knife and knife to fresh agar, which was comparable to
120 planktonic cells (**Figure 2a-b**), indicating the importance of exopolysaccharides in
121 biofilm transfer. In contrast, eDNA was less important in *P. aeruginosa* biofilms (**Figure**
122 **2a-b**), probably because of its lower composition in the biofilm matrix (Sutherland,
123 2001).
124

125 **Water content facilitates higher biofilm transfer.**

126

127 Various foods have different water content, which raises the question if the water
128 content plays a role in the biofilm transfer. We first tested experimentally different
129 concentrations of agar from the lowest (0.375%, indicating highest water content) to the
130 highest (3%, indicating lowest water content), where we observed higher transfer of
131 biofilm-dispersed cells than planktonic cells consistently across different water content
132 (**Figure 3a-b**). However, there was a significant increase in the bacterial adherence on
133 knives from 0.375% to 3% LB agar, indicating that biofilm-dispersed cells tend to be
134 transferred better on high water content.
135 Our findings were also corroborated using food, namely potato slices, where raw potato
136 has a higher water content than cooked potato (Decker and Ferruzzi, 2013). We found
137 that bacterial transfer on raw potato was higher than cooked potato (**Figure 3c-d**). This
138 implied that food with a higher water content could be more prone to biofilm
139 contamination and transfer than drier foods. This supported the previous findings that
140 water content played a significant role in bacterial transfer between food and non-food
141 surfaces (Miranda and Schaffner, 2016).

142

143 **Combination of detergent and mechanical wiping significantly reduced biofilm**
144 **transfer.**

145

146 Lastly, we aim to evaluate the use of detergent, specifically the food-grade Tween 20
147 (polysorbate 20) (Kimura et al., 1982; Nguyen-The and Lund, 1992; Vatić et al., 2020),

148 to eliminate bacterial cells on the cutting tool, to reduce cross-contamination across
149 foods. Previous studies had shown the use of detergents to treat biofilms on abiotic
150 surfaces (Tsiaprazi-Stamou et al., 2019). As for mechanical wiping, we found its
151 efficiency in reducing bacterial numbers on the knife and surfaces but was insufficient to
152 eliminate the bacteria completely (**Supplementary Figure 4**). Hence, the combinatorial
153 treatment of mechanical wiping and detergent of cutting tool significantly improved the
154 cleaning of cutting tool, resulting in poor bacterial transfer across media (**Figure 4a-b**).
155
156

157 **Discussion:**

158

159 Pathogens form biofilms which can attach any abiotic and biotic surfaces, which confers
160 several survival benefits, such as protection from predators and better nutrient
161 availability. To ensure continuity of the species, biofilm dispersal occurs to allow
162 bacteria to leave the biofilms during periods of stress or starvation and colonize fresh
163 areas. Mechanical methods, such as shearing and sloughing (Kaplan, 2010), can also
164 cause biofilm dispersal, where we showed that biofilm-dispersed cells released via
165 mechanical dislodging (cutting) disseminate more efficiently than planktonic cells.

166

167 Due to the presence of sticky matrix which comprises of different biofilm matrix
168 components, biofilms are transferred easily across biotic and abiotic surfaces, as
169 compared to planktonic cells. We showed that biofilm matrix exopolysaccharides in
170 general were most important in the bacterial transfer via mechanical dislodging
171 phenotype. The exopolysaccharides were major components in biofilm matrix
172 (Wickramasinghe et al., 2020), where they were previously shown to have several
173 functions, such as preventing antibiotic penetration (Ciofu et al., 2017), resisting
174 oxidative stress and immune clearance (Chua et al., 2016), and impeding predator
175 motility (Chan et al., 2021). On the other hand, while we showed that eDNA was not as
176 important as exopolysaccharides, there could be other bacterial species which primarily
177 incorporate eDNA as its biofilm matrix (Aung et al., 2016; Deng et al., 2021), which
178 warrants the need to test the phenotype on more bacterial species. Understanding the

179 main components of the biofilm matrix will enable us to develop specific targets against
180 the biofilm matrix.
181
182 Our phenotype is applicable to food cross-contamination. Cutting tools are highly
183 susceptible to cross-contamination when shredding or cutting foods. However, while
184 effective against planktonic cells, we found that simple sanitization by using detergents
185 or mechanical wiping is insufficient in clearing the biofilm-released cells. This indicated
186 that biofilm-released cells remained highly recalcitrant to simple chemical removal.
187 Since further treatments either not realistic for everyday purposes or expensive,
188 mechanical disruption of biofilms via wiping with detergent is a simple, cheap, and
189 efficient way to drastically reduce the bacterial biofilm numbers. Alternatively,
190 hydrophobic repellents or anti-biofilm agents may be required to improve the elimination
191 of biofilms or repulsion of attaching bacterial cells to surfaces (Yu and Chua, 2020). For
192 example, Sharklet is a commercial product based on shark skin-like engineered surface
193 microtopography, which can prevent bacterial attachment on surfaces (Chung et al.,
194 2007). Natural anti-biofilm agents, such as vanillin from vanilla and ajoene from garlic
195 (Jakobsen et al., 2012; Mok et al., 2020), could also be incorporated onto the culinary
196 surfaces for inhibiting biofilm formation by foodborne pathogens.
197
198 In summary, our work provided insights into biofilm-released bacteria from mechanical
199 dislodging which disseminate better than planktonic bacteria. Hence, it is important for
200 kitchens and food processing plants to properly sanitize these cutting tools frequently.

Materials and Methods:

- Bacterial strains and growth conditions:

The *P. aeruginosa* strains used in this project are listed in Table 1. Mutant strains of *P. aeruginosa* are comprised of varying profiles of biofilm compositions. Wild-type strains of *S. aureus*, *V. cholerae*, and *S. enterica* spp. Typhimurium are also used in this study. All bacterial strains were inoculated in 2 ml of Lysogeny broth (LB) (Becton, Dickinson and Company, USA) at 37 ℃, shaken at 200 rpm for 16 hrs.

- Preparation of medium:

The experimental medium used in this project includes 0.375, 0.75, 1.5, and 3% LB agar, raw, and cooked potato.

0.375, 0.75, 1.5, and 3% (w/v) LB agar were prepared by mixing LB broth and Bacto-agar (Becton, Dickinson and Company, USA). 15 ml of LB agar was poured into the petri dish (SPL, Korea) consistently for each experiment throughout the project. For potato studies, a fresh potato of dimensions of 1.6 cm (l) X 0.4 cm (w) X 0.4 cm (h) was used for experiment. Raw potato slices were cooked in a microwave oven for 1 minute to achieve cooking.

- Cultivation of bacteria on media:

Bacteria of various species and mutants were cultivated on LB agar plate of various agar concentrations (0.375, 0.75, 1.5, and 3% w/v agarose) or potato (raw and cooked).

224 For biofilm formation, overnight cultures were washed and diluted in 0.9% (w/v) NaCl
225 saline solution (Sigma-Alrich, Germany) to a final concentration of 10^3 cells/ ml, followed
226 by spreading of 100 ul diluted cultures on the media surface and cultivation of biofilm
227 lawn at 37℃ for 24 hours, for achieving a final concentration of 10^9 cells/ ml.
228
229 For planktonic cells, the overnight cultures were washed and diluted in 0.9% (w/v) NaCl
230 saline solution to a final concentration of 10^6 cells/ ml, followed by spreading of 100 ul
231 diluted cultures on the media surface. The liquid was allowed to dry briefly on the surface
232 so that the planktonic cells will be deposited on the media surface.
233
234 *- Transfer of bacterial cells from inoculated medium to sterile medium via cutting tools:*
235 Prior to start of experiment, the knives were wiped with 70% ethanol (v/v) (Sigma-Alrich,
236 Germany) and then air-dried briefly before every use. The cutting tools made of
237 ceramic, plastic or stainless steel were used to cut a 1 cm slit across the planktonic or
238 biofilm cells on the media at a near-horizontal angle. The cutting tool with the attached
239 bacterial cells was then transferred to a fresh media surface for cutting a 1-cm slit. A
240 similar experiment was adopted for raw and cooked potato slices, where a 1-cm slit was
241 cut across the potato slice (size 1.6 cm (l) X 0.4 cm (w) X 0.4 cm (h)) using a ceramic knife
242 and transferred to a fresh media surface with the next 1-cm slit.
243
244 *- Cleaning and decontaminating of the cutting tool via mechanical disruption (wiping)*
245 *and detergent:*

246 The Tween-20 detergent (Sigma-Alrich, Germany) was first sterilized with 0.2 μm
247 membrane filters and prepared at concentrations of 0.01%, 0.1%, 1% and 10% (v/v) in
248 sterile ddH_2O. After the cutting tool was used to cut the original contaminated surface, a
249 clean C-fold towel wetted with detergent was employed to wipe the cutting tool in a
250 unidirectional manner for 3 times. The cutting tool was then dipped gently into sterile
251 saline for 5 times to thoroughly remove the detached bacterial cells and excess
252 detergent. The cutting tool was subsequently used to cut the fresh media surface for
253 bacterial quantification.
254
255 - *Bacterial quantification by colony-forming units (CFU)*
256 To quantify the bacterial cells on the original contaminated media, knife, and fresh
257 media, we first retrieved the cells by cutting the surrounding media around the slit with
258 standardized dimensions of 1.6 cm (l) X 0.4 cm (w) X 0.4 cm (h) and dislodging the
259 bacteria from the medium surface in 1ml 0.9% (w/v) NaCl saline by sonication in ice
260 bath for 15mins. The similar procedure was adopted for the cutting tool, where it was
261 placed in saline and sonicated in ice bath for 15mins. The saline containing the released
262 bacteria was subsequently homogenized by vortex mixing for 15 s.
263 As previously described (Liu et al., 2021), the cells suspensions were diluted serially in
264 saline and transferred to LBA agar plates (5 technical replicates) for incubation at 37 °C
265 for 16 hrs. Colonies that grew on the petri dishes were enumerated and tabulated with
266 CFU ml^{-1} = colony number X dilution factor X volume.
267 Appropriate transfer rates were calculated as previously described (Chen et al., 2001),
268 in the following equations:

269 [1] Transfer from contaminated agar to knife:

270 Transfer rate (%) = (CFU on knife/ CFU on contaminated agar) X 100

271 [2] Transfer from knife to fresh agar:

272 Transfer rate (%) = (CFU on fresh agar/ CFU on knife) X 100

273 Experiments were performed in biological triplicates, and the results are shown as the

274 mean ± s.d.

275

276 - *Imaging of bacterial cells on surfaces by confocal microscopy*

277 As previously described (Liao et al., 2021), *gfp*-tagged bacteria attached onto the

278 contaminated medium and fresh medium were imaged by Confocal Microscope (Leica

279 TCS SP8 MP, Germany) (both brightfield and GFP fluorescence field using 488 nm

280 laser (Ex: 495 nm; Em: 515 nm)) with 10X objective and Z-stack function. At least 5

281 images were captured and processed by ImageJ, where representative image was used

282 for presentation.

283

284 - *Statistical analysis*

285 Independent experiments (n=3) were performed in technical triplicate, where one-way

286 ANOVA and Student's t-tests were used to establish statistical significance and the

287 results were shown as the mean ± s.d.

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291 Laboratory of Chemical Biology and Drug Discovery Fund (1-BBX8).

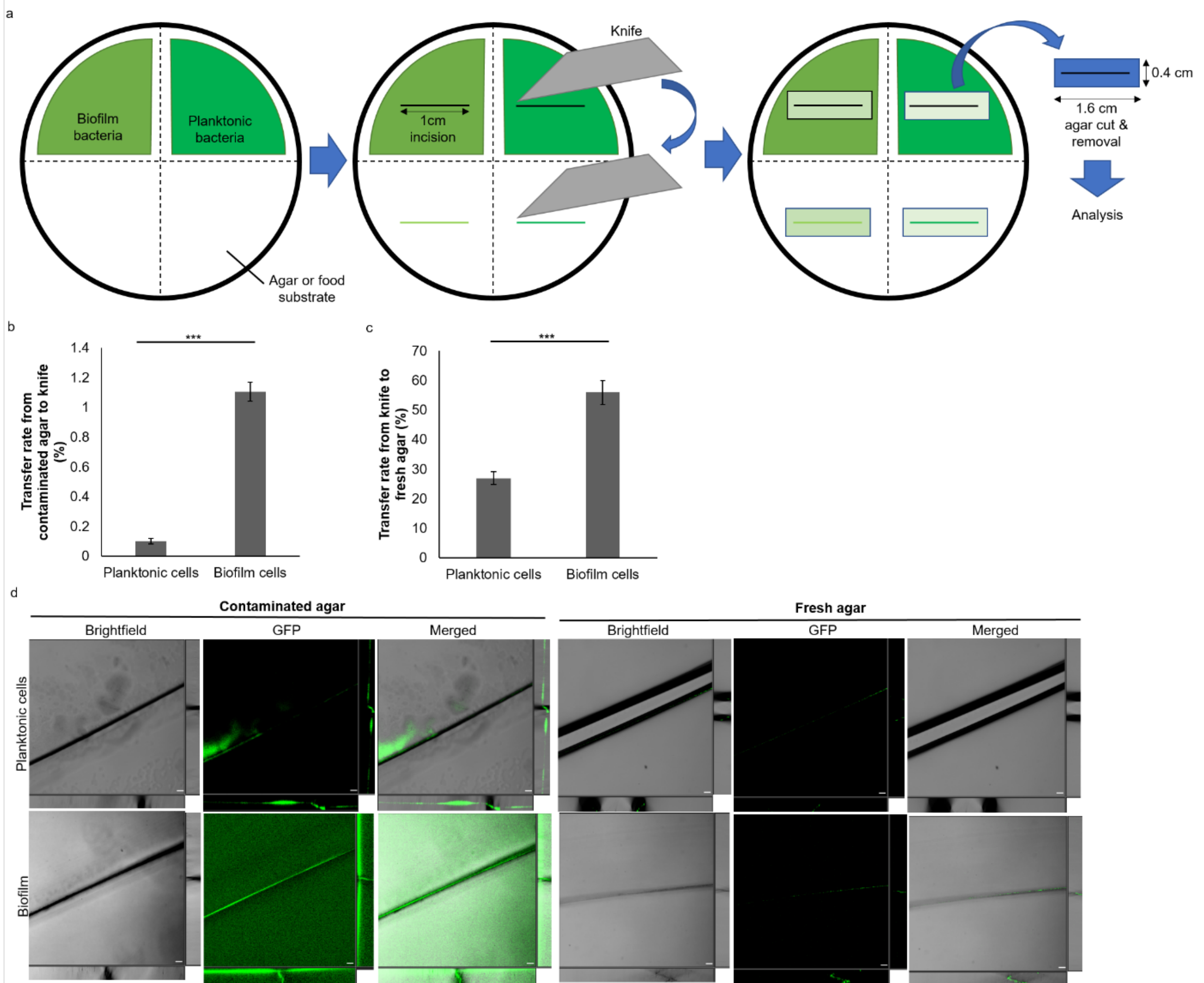
292

293 **Competing interests:**

294 The authors declare no competing financial interests.

295

296 **Figures:**



297

298 **Figure 1: Biofilms are easily transferred to fresh media by cutting than planktonic**

299 **bacteria**. (a) Scheme depicting the cross-contamination of surfaces using cutting tools.

300 (b) Transfer ratio of bacteria on knife to bacteria from contaminated agar. (c) Transfer

301 ratio of bacteria on fresh agar to bacteria from contaminated agar. Means and s.d. from

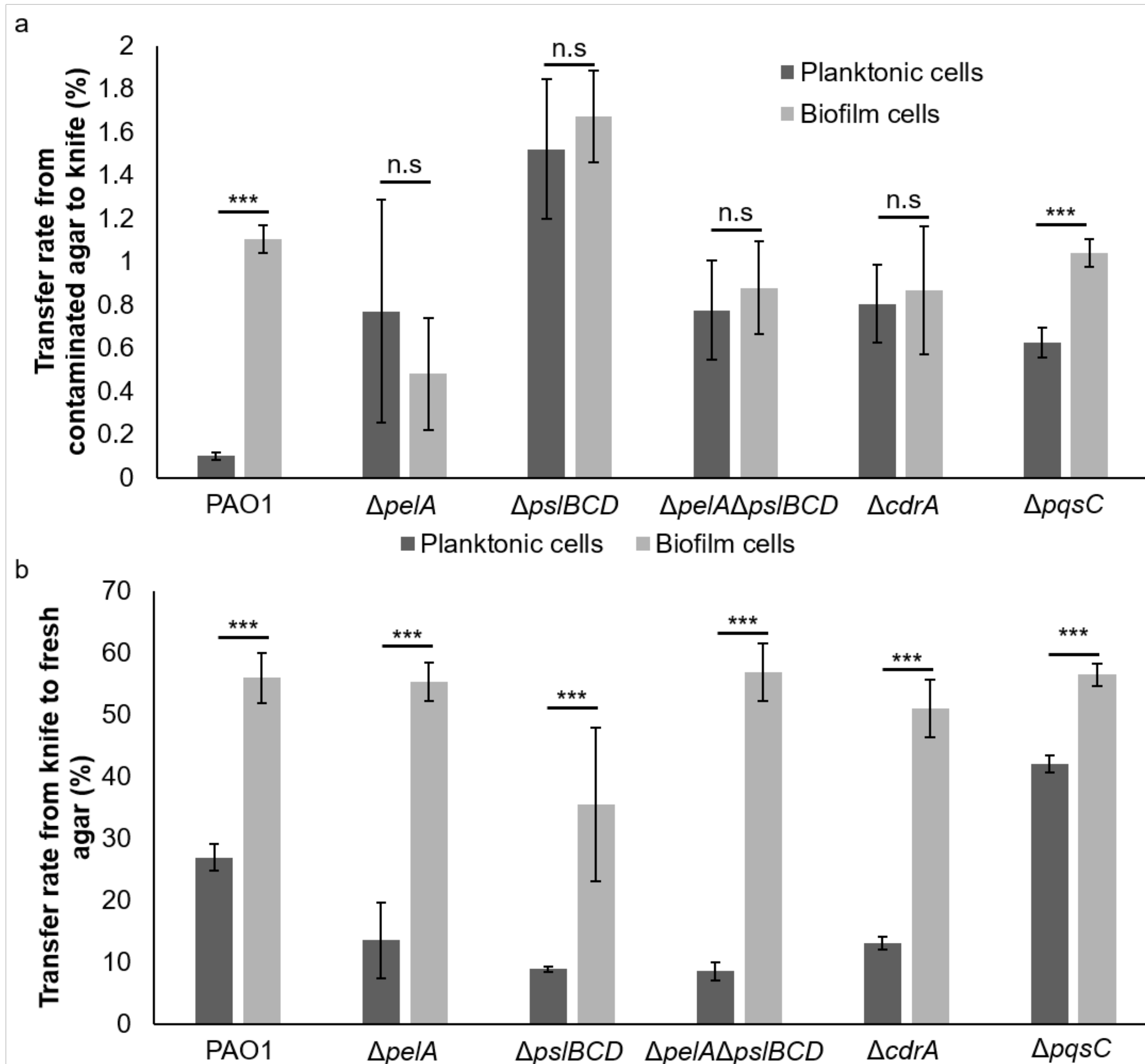
302 triplicate experiments are shown. ***$P < 0.001$,1-way ANOVA. (d) Representative

303 images of *gfp*-tagged *P. aeruginosa* on cut surfaces from original contaminated agar

304 and fresh agar. Scale bar: 100 µm.

305

306

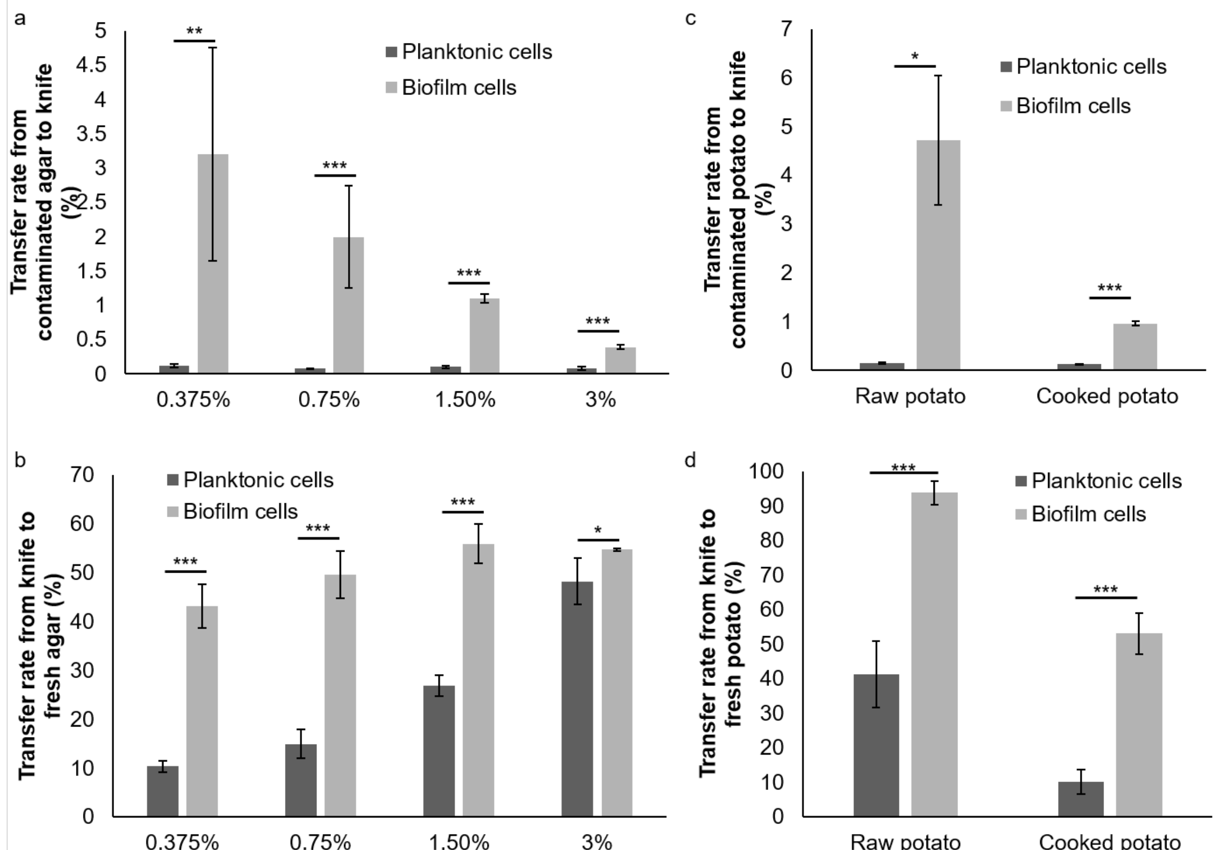


307 **Figure 2: Biofilm matrix is important to efficient biofilm transfer via cutting, where**
308 **biofilm matrix mutants cannot be transferred effectively as compared to wild-type**
309 **biofilms.** (a) Transfer ratio of bacteria on cutting tool to bacteria from contaminated
310 agar. (b) Transfer ratio of bacteria on fresh agar to bacteria from contaminated agar.
311 Means and s.d. from triplicate experiments are shown. *P < 0.05, ***P < 0.001, n.s: not
312 significant, 1-way ANOVA.

313

314

315



316 **Figure 3: Effect of water content on biofilm transfer.** (a) Transfer ratio of bacteria on
317 knife to bacteria from contaminated agar. (b) Transfer ratio of bacteria on fresh agar to
318 bacteria from contaminated agar. (c) Transfer ratio of bacteria on cutting tool to bacteria
319 from contaminated potato slices. (d) Transfer ratio of bacteria on fresh potato slices to
320 bacteria from contaminated potato slices.

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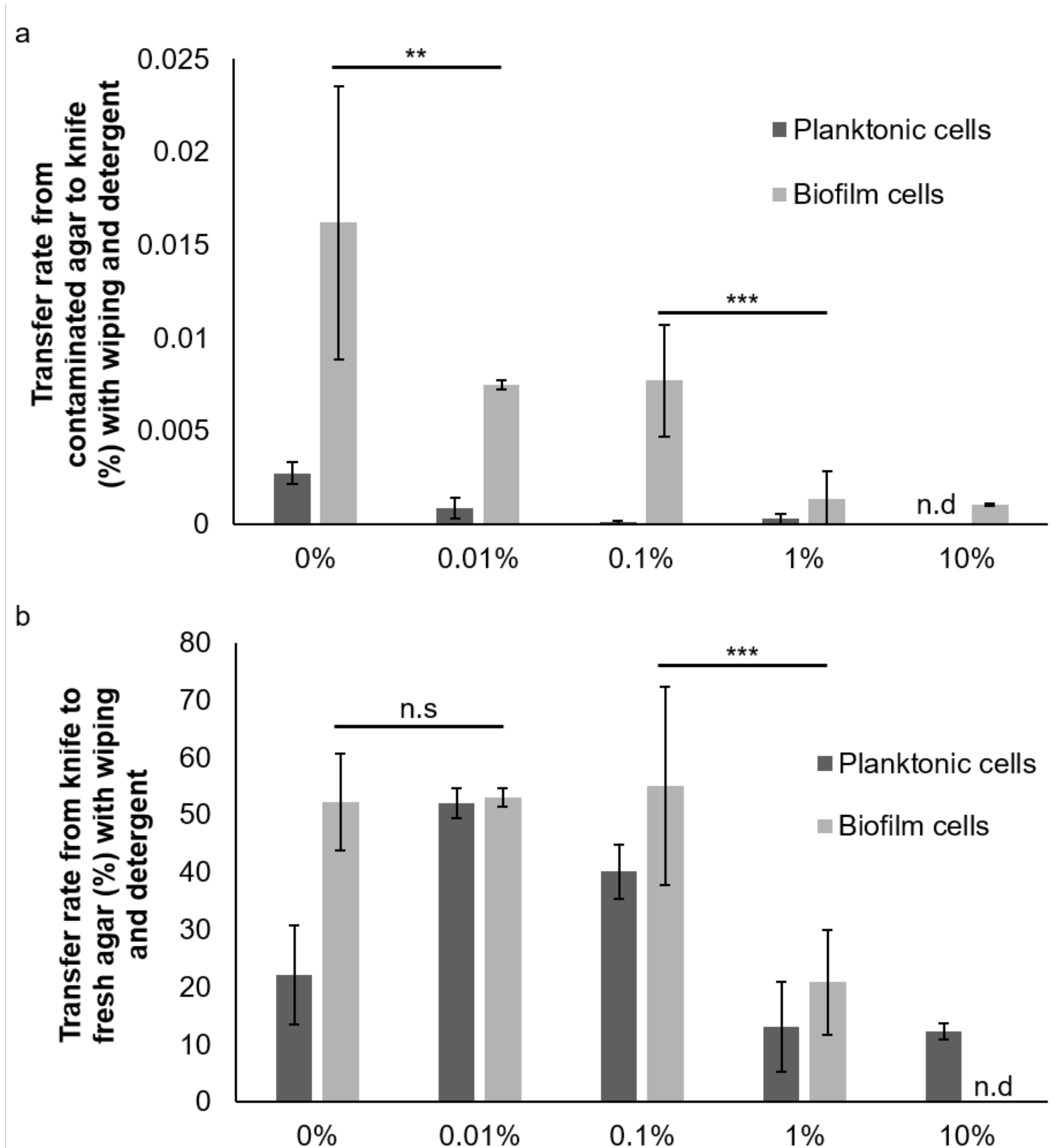

323 **Figure 4: Effect of washing knife with a combinatorial treatment of mechanical**
324 **wiping and detergent before recutting.** (a) Transfer ratio of bacteria on cutting tool to
325 bacteria from contaminated agar. (b) Transfer ratio of bacteria on fresh agar to bacteria
326 from contaminated agar.

327

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