Cleaning and disinfection of biofilms composed of *Listeria monocytogenes* and background microbiota from meat processing surfaces

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ABSTRACT

Surfaces of food processing premises are exposed to regular cleaning and disinfection (C&D) regimes, using biocides that are highly effective against bacteria growing as planktonic cells. However, bacteria growing in surface associated communities (biofilms) are typically more tolerant towards C&D than their individual free cells counterparts, and survival of pathogens such as *Listeria monocytogenes* may be affected by interspecies interactions within biofilms. In this study, *Pseudomonas* and *Acinetobacter* were the most frequently isolated genera surviving on conveyor belts subjected to C&D in meat processing plants. In the laboratory, *Pseudomonas*, *Acinetobacter* and *L. monocytogenes* dominated the community both in suspensions and in biofilms formed on conveyor belts, when cultures were inoculated with eleven-genera cocktails of representative bacterial strains from the identified background flora. When biofilms were exposed to daily C&D cycles, mimicking treatments used in food industry, the levels of *Acinetobacter* and *Pseudomonas mandelii* diminished, and biofilms were instead dominated by *Pseudomonas putida* (65-76%), *Pseudomonas fluorescens* (11-15%) and *L. monocytogenes* (3-11%). The dominance of certain species after daily C&D correlated with high planktonic growth rates at 12°C and tolerance to C&D. In single-species biofilms, *L. monocytogenes* developed higher tolerance to C&D over time, both for the peracetic acid and quaternary ammonium disinfectant, indicating that a broad-spectrum mechanism was involved. Survival after C&D appeared to be a common property of *L. monocytogenes* strains, as both persistent and sporadic subtypes showed equal survival in complex biofilms. Biofilms established preferentially

in surface irregularities of conveyor belts, potentially constituting harborage sites for persistent contamination.

IMPORTANCE

In food industry, efficient production hygiene is a key measure to avoid accumulation of spoilage bacteria and eliminate pathogens. Persistence of bacteria is however a withstanding problem in food processing environments. This study demonstrated that environmental bacteria can survive foam cleaning and disinfection (C&D) at user concentrations in the industrial environment. The phenomenon was replicated in laboratory experiments. Important characteristics of persisting bacteria were high growth rate at low temperature, tolerance to the cleaning agent and ability to form biofilm. This study also supports other recent research suggesting that strain-to-strain variation cannot explain why certain subtypes of *Listeria monocytogenes* persist in food processing environments while others are found only sporadically. The present investigation highlights the failure of regular C&D and a need for research on improved agents efficiently detaching the biofilm matrix.

INTRODUCTION

Food production premises are regularly subjected to cleaning and disinfection (C&D) regimes designed to reduce bacterial load and eliminate pathogens. Peracetic acid (PAA) and quaternary ammonium compounds (QAC) such as benzalkonium chloride are widely used as disinfectants in the food industry and in healthcare facilities. Disinfectants are agents that have multiple targets in the cell, and typically kill bacteria by disruption of the bacterial membrane (1). The use of

chemical disinfectants in food processing environments is usually based on their efficacy in tests performed with planktonic bacteria (2). However, in natural and industrial environments, bacteria often grow as biofilms, which are complex and structured microbial communities encased in a self-produced protective extracellular matrix composed of polysaccharides, proteins and/or extracellular DNA. The formation of biofilms is important for microbial survival in the food industry, and cells in biofilms typically exhibit increased tolerance towards antimicrobial agents compared with their planktonic counterparts (3, 4). Possible mechanisms contributing to the low efficacy of conventional biocides on biofilms include diffusion-reaction limitation associated with the biofilm matrix, slow growth and development of persister cell subpopulations (4).

The microbiota found in food processing plant surfaces after C&D is commonly reported to be diverse and include foodborne pathogens and food spoilage bacteria. Predominant genera in meat processing plants after C&D include *Pseudomonas*, *Acinetobacter*, *Staphylococcus* and *Serratia* (5-7). One of the pathogens regularly encountered in such environments is *Listeria monocytogenes*, which causes the life-threatening disease listeriosis. This bacterium poses a significant food safety challenge given its wide distribution in nature and its ability to grow at refrigeration temperatures and to survive and persist on equipment in food processing environments. Contamination of food products with *L. monocytogenes* mainly occurs in the food production environment, and is a concern especially with regard to ready-to-eat (RTE) products such as cold meat cuts. Transfer of *L. monocytogenes* from food contact surfaces such

71 as conveyor belts onto processed food products have been documented, and in some cases
72 shown to result in outbreaks of listeriosis (8, 9).

73 Certain strains of *L. monocytogenes* can establish in the production environment and persist for
74 months or even years, especially in humid areas and areas where C&D is difficult. Persistent
75 strains of *L. monocytogenes* often belong to certain molecular subtypes, while other subtypes
76 are found only sporadically (10-14). Several studies have investigated whether phenotypic traits
77 such as the ability to form biofilms and survive biocide action may be responsible for the
78 prolonged persistence of certain strains on food processing plant surfaces (15-18). Individual
79 strains of *L. monocytogenes* have been shown to vary in their ability to form biofilms (19, 20)
80 and differ in their tolerance towards disinfectants (21, 22). However, no single genetic
81 determinant or individual trait responsible for *L. monocytogenes* persistence has been
82 identified, and it is now generally thought that the perceived persistence of certain subtypes of
83 *L. monocytogenes* is due to a complex combination of factors (13, 14).

84 The resident background microflora is recognized to play an important role with respect to
85 protecting and sheltering pathogenic strains within food processing environments. Weak biofilm
86 formers can for instance improve their survival by joining a multispecies biofilm (23-25).
87 Additionally, it appears that biofilms composed of multiple genera are generally less susceptible
88 to biocide action than their single-species counterparts (4, 23, 26, 27). For example, under most
89 conditions, dual species biofilms of *L. monocytogenes* and *Lactobacillus plantarum* were more
90 tolerant to benzalkonium chloride and PAA than were the corresponding single species biofilms
91 (28). Nevertheless, specific bacterial interactions, which include competition, coaggregation and

metabolic cross-feeding, may have variable effects on the survival of individual biofilm community members (23). Growth of *L. monocytogenes* in dual-species biofilms with representative strains from food production environments has for instance resulted in both enhanced and reduced cell numbers of *L. monocytogenes* (29). It is, however, not clear to what extent these effects vary between strains or subtypes of *L. monocytogenes*, or how different *L. monocytogenes* strains survive in more complex multigenera biofilms subject to conditions similar to those found in food industry.

The purpose of this study was to examine biofilm formation and survival of strains belonging to bacterial genera commonly isolated from conveyor belts in meat processing environments, under conditions simulating those encountered in these environments. This included an assessment of the efficacy of C&D under relevant conditions, and an examination of how the background microbiota may affect growth and survival of persistent and sporadic *L. monocytogenes* subtypes in biofilms exposed to C&D. Initially, the microbiota surviving C&D of conveyor belts in meat processing plants was identified. An experimental biofilm model system was then set up using conditions realistic for food industry, including growth on coupons cut from conveyor belt material and exposure to daily cycles of C&D. Biofilms composed of *L. monocytogenes* strains were compared with complex multigenera biofilms inoculated with both *L. monocytogenes* and selected strains dominating the bacterial flora identified in meat processing environments. The development of the biofilm microbiota was investigated using viability counting, amplicon sequencing and imaging techniques.

RESULTS

Identification of microbiota on conveyor belts in meat processing plants. Sampling of nine conveyor belts after sanitation in two meat processing plants resulted in identification of a total of 121 isolates from a total of 22 genera (Table 1). Eight genera were common for both plants, but overall, the microbiota after sanitation differed between plants and between single conveyor belts. For two of the six conveyor belts sampled in Plant A, the bacterial numbers were very low and four or less isolates were collected (conveyors 4 and 5). For conveyors with higher bacterial numbers, *Pseudomonas* was most frequently isolated and dominated alone in one sample, together with *Psychrobacter* in another, and with *Acinetobacter* on a third conveyor belt. For one conveyor belt, which was associated with a permanent *L. monocytogenes* (MF5377) reoccurrence, a diverse microbiota was found in which *Microbacterium* dominated together with *Epilithonimonas*. In Plant B, *Sphingomonas* dominated together with *Rhodococcus* on one conveyor and with *Acinetobacter* on another. Only five isolates were collected from the third conveyor belt. A total of 16 isolates were selected for the present study, representing the most dominant bacteria (Table 2).

Three *Pseudomonas* sp. and two *Acinetobacter* sp. were subjected to whole genome sequencing and phylogenetic analysis to further determine their taxonomic status. This analysis showed that strain MF6396 belonged to the *Pseudomonas putida* group, and that strains MF6394 and MF4836 belonged to the *Pseudomonas fluorescens* and *Pseudomonas mandelii* subgroups, respectively, within the *P. fluorescens* complex (Fig. S1 in the Supplemental Material). Thus all three strains belong to the *P. fluorescens* lineage. For simplicity, these strains are referred to as

133 *P. putida* MF6396, *P. fluorescens* MF6394 and *P. mandelii* MF4836 in the remainder of this text.
134 Both *Acinetobacter* strains included in the experiments (MF4640 and MF4642) were determined
135 to belong to the species *A. johnsonii* using *in silico* multilocus sequence typing (MLST) (Fig. S2 in
136 the Supplemental Material).

137 ***Pseudomonas* and *Acinetobacter* dominated in laboratory multigenera biofilms.** A
138 biofilm model system was set up to examine biofilm formation and survival under conditions
139 simulating food production environments. Biofilms were grown on conveyor belt coupons
140 placed vertically in 24-well plates with BHI broth at 12°C, which is a temperature typically found
141 in Norwegian meat processing facilities. In addition to the 16 strains from the background
142 microbiota found on conveyors in meat processing plants (described above), seven *L.*
143 *monocytogenes* strains belonging to different phylogenetic clusters were selected for inclusion
144 in biofilm experiments (according to selection criteria in Materials and Methods section). Four
145 belonged to MLST sequence types (STs) responsible for persistent contaminations in Norwegian
146 food processing plants, while three strains belonged to STs which were only sporadically
147 encountered in Norwegian food industry (Table 2) (22). Coupons were inoculated with a
148 suspension of either the 16 background microbiota strains plus the seven *L. monocytogenes*
149 strains (referred to as multigenera biofilms), or with only the seven *L. monocytogenes* strains (*L.*
150 *monocytogenes* biofilms). The biofilms were allowed to develop for four days and subsequently
151 subjected to C&D on Days 4 to 7, using a chlorinated alkaline cleaning agent (Alkalifoam) and
152 disinfection with either a QAC- or PAA-based disinfectant, at user concentrations recommended
153 by the manufacturers. Wells containing multigenera biofilms usually contained a floating pellicle

154 that was attached to the coupon at the air-liquid interface. Visible biofilm deposits were
155 generally observed in this zone of the coupons after C&D.

156 The development of the microbiota in the multigenera biofilms was investigated using 16S rRNA
157 amplicon sequencing. The results showed that after four days of biofilm growth, one of the *A.*
158 *johnsonii* strains (MF4640) dominated the biofilm, while after seven days of growth, the *P.*
159 *putida* strain (MF6396) had taken over as the dominant strain. The proportion of *L.*
160 *monocytogenes* in the multigenera biofilm was higher on Day 7 than on Day 4 (Fig. 1A). To
161 investigate whether the shift in microbiota from Day 4 to Day 7 was only due to establishment
162 of a more mature biofilm, or also affected by the C&D cycles, new experiments were conducted
163 in which only the three dominating *Pseudomonas* spp. strains and *A. johnsonii* strain MF4640
164 were included. Here, coupons that were rinsed daily with H_2O were included in addition to
165 coupons treated with C&D agents. The results presented in Fig. 1B show that the bacterial strain
166 composition identified on coupons subjected to C&D in these two additional experiments were
167 similar to those obtained in the first three experiments in which biofilms were inoculated with
168 all 16 background microbiota strains (Fig. 1A and Fig. S3 in the Supplemental Material).
169 However, in the absence of C&D, no significant shift in the microbiota composition was
170 observed from Day 4 to Day 7, and the *A. johnsonii* strain dominated, followed by *P. putida*. This
171 indicated that daily exposure to C&D selected for *P. putida*, *P. fluorescens* and *L. monocytogenes*
172 and almost eliminated the *P. mandelii* and *A. johnsonii* strains.

173 **No selection between different *L. monocytogenes* strains was observed in biofilms.** To
174 determine whether the different *L. monocytogenes* strains had different fitness during growth

in biofilms subjected to C&D, strain identification of single colonies collected after Day 7 of biofilm growth were performed by sequencing of the *dapE* MLST allele (Fig. 2A and Fig. S4 in the Supplemental Material). The frequencies of each strain across all tested samples ranged from 5% for MF5378, to 28% for MF5360. The four strains belonging to persistent subtypes had an overall frequency of 51% across all samples, indicating that these strains did not have a greater ability to survive in biofilms exposed to C&D than strains belonging to sporadic subtypes. No evidence for selection between different *L. monocytogenes* strains was observed, neither in the multigenera biofilms where *L. monocytogenes* was grown in the presence of 16 background flora strains, nor in biofilms containing *L. monocytogenes* only.

Strains dominating in the multigenera biofilm showed high growth rates in planktonic culture. The relative amounts of each bacterial strain in planktonic cultures inoculated with the same bacteria as were used in the multigenera biofilm experiments is shown in Fig. 1C. As in the biofilm experiments, the bacterial composition developed towards *A. johnsonii*, *Pseudomonas* spp. and *L. monocytogenes*. In contrast to during growth in biofilm conditions, however, both *A. johnsonii* strains (MF4640 and MF4642) seemed to compete equally well under planktonic culture conditions. Similarly, the three *Pseudomonas* spp. strains were in approximately equal proportions in the planktonic cultures, while in the biofilms, there was significantly more of *P. putida* MF6396 than of *P. fluorescens* MF6394 and especially *P. mandelii* MF4836.

All seven *L. monocytogenes* strains were retained in approximately equal amounts when grown together in planktonic culture, both when they were grown alone and when they were grown

195 together with the 16 background microbiota strains (Fig. 2B and Fig. S4 in the Supplemental
196 Material). The proportion of each strain present in the cultures containing only *L.*
197 *monocytogenes*, determined using *dapE* amplicon sequencing, ranged from on average 9%
198 (MF5376/ST7) to 21% (MF5377/ST8) after 72 h of growth. When the seven *L. monocytogenes*
199 strains were grown together with the 16 background microbiota strains, the proportion of each
200 *L. monocytogenes* strain after 72 h ranged from 12% (MF5376/ST7 and MF5634/ST121) to 18%
201 (MF5377/ST8). These results indicated that during planktonic growth at 12°C, none of the seven
202 *L. monocytogenes* strains appeared to have a growth advantage allowing them to outcompete
203 any of the other strains.

204 When the individual strains were grown in separate wells in a Bioscreen C instrument (Fig. 3 and
205 Table S1 in the Supplemental Material), the largest maximal growth rates during the exponential
206 phase of growth was attained by *P. fluorescens* MF6394, followed by *P. mandelii* MF4836, the
207 two *A. johnsonii* strains and then *P. putida* MF6396. The cultures containing *Pseudomonas*
208 strains ultimately reached higher values of OD_{600} than the *Acinetobacter* cultures. Other strains
209 with high growth rates were the seven *L. monocytogenes* strains (which all had similar growth
210 curves) and *Epilithonimonas* strain MF6392, followed by the *Psychrobacter* and *Microbacterium*
211 strains (MF4641 and MF4634). It thus seems like the strains showing rapid planktonic growth at
212 12°C in BHI culture medium are highly competitive in the biofilms grown on conveyor belt
213 coupons.

The *Pseudomonas* genomes contained different sets of known biofilm-associated genes. The difference in competitiveness between the three *Pseudomonas* strains and between the two *A. johnsonii* strains in planktonic culture compared to growth in the conveyor belt biofilm model (Fig. 1) could possibly be due to differences in the ability to form biofilms. Therefore, the *Pseudomonas* spp. and *A. johnsonii* genomes were screened for known biofilm-associated genes using BLAST analysis. All three *Pseudomonas* strains contained the *alg* operon required for alginate synthesis, and homologs to the *lapABCD* and *lapG* genes required for expression of the large surface protein LapA on the cell surface. The genes responsible for Pel, Psl, and cellulose synthesis were however each only present in one of the three strains: *P. fluorescens* MF6394 contained a *psl* operon, *P. mandelii* MF4836 contained a *pel* operon, while a homolog to the *wss* operon required for cellulose synthesis was present in *P. putida* MF6396. It thus appears that all three strains harbor genetic factors enabling biofilm formation (Table S2 in the Supplemental Material). With respect to the two *A. johnsonii* strains, not much is known about biofilm formation in non-*baumanii Acinetobacter* strains, and no homologs to genes shown to be involved in biofilm formation in *Acinetobacter baumanii* were identified in the genomes of the two *A. johnsonii* strains employed in the current study. The two strains did however have different genome sizes, as the genome of *A. johnsonii* MF4640 was 13% larger than that of the 3.36 Mbp large genome of strain MF4642. A large portion of the additional genetic material in MF4640 appears to constitute plasmids and other mobile genetic elements.

The sanitation regime was inefficient at killing bacteria in conveyor belt biofilms. To assess sanitation efficacy in the biofilm model system, the total number of colony-forming units

(CFUs) in biofilms growing on conveyor belt coupons was determined both before and after coupons were subjected to C&D.

After the initial four days of biofilm development, the cell densities in multigenera biofilms reached about 1×10^8 CFUs per coupon (3 cm^2 surface area). Coupons were then subjected to daily cycles of C&D for three days, and sampled again on Day 7 after allowing 24 h of regrowth after the last C&D cycle. Control coupons were rinsed with sterile deionized water (H_2O) every day. There was no significant difference in cell densities on coupons with multigenera biofilms sampled prior to C&D on Day 4 and Day 7, regardless of whether coupons had been treated with QAC, PAA, or rinsed in H_2O (P>0.05; Fig. 4, grey bars). Thus neither the QAC- nor PAA-based C&D regimes altered the total amount of biofilm on conveyor belt material present 24 h after C&D treatment.

However, while the total number of CFUs on each coupon in the multigenera biofilm was similar in all tested samples, the amount of *L. monocytogenes* in the biofilm increased about tenfold from Day 4 to Day 7 (Fig. 4, yellow bars). The fraction of *L. monocytogenes* in the multigenera biofilms increased from 2.3% (SE±1.1%) on Day 4 to 9% (SE±2%), 18% (SE±4%) and 32% (SE±7%) in the H_2O-rinsed, QAC-treated, and PAA-treated biofilms, respectively, harvested on Day 7. In the biofilms where *L. monocytogenes* were grown alone, however, there was no statistically significant difference in *L. monocytogenes* counts per coupon between Days 4 and 7 (P>0.05; Fig. 4, green bars), with around 2×10^7 CFUs per coupon on both days and across the different treatments.

255 When the total numbers of CFUs per coupon before and after C&D were compared, between
256 0.6 and 0.9 $\log_{10}$ reductions in total CFUs were observed on Day 4 and Day 7, respectively, for
257 coupons harboring multigenera biofilms (Fig. 5, grey bars). The difference in $\log_{10}$ reductions
258 between treatments or day of sampling was not statistically significant (P>0.05).

259 The $\log_{10}$ reduction for the *L. monocytogenes* component of the multigenera biofilm was
260 significantly lower than the $\log_{10}$ reduction in total CFUs per coupon when the Day 4 coupons
261 were treated with PAA disinfection (0.6 vs. 0.9 $\log_{10}$ reduction; P=0.04). For the other
262 treatments (QAC-treatment on Days 4 and 7, and PAA-treatment on Day 7), there was no
263 difference in survival of the flora strains and the *L. monocytogenes* strains in the multigenera
264 biofilm upon C&D (P>0.05; Fig. 5, compare grey and yellow bars). This indicates that the
265 proportion of *L. monocytogenes* cells in the biofilm was relatively stable during a cycle of C&D.

266 For the *L. monocytogenes* biofilms, on Day 4, the reduction in CFUs per coupon upon sanitation
267 treatment was about the same as for the multispecies biofilms. On Day 7, however, there was
268 almost no reduction in bacterial numbers upon C&D, with average reductions in cell numbers of
269 only 0.13 and 0.26 $\log_{10}$ CFUs per coupon upon QAC- and PAA-treatment of the biofilms,
270 respectively (Fig. 5, green bars).

271 Overall, these experiments indicated that biofilms on conveyor belt materials were not
272 eliminated when exposed to a C&D regime relevant for the food industry. Little or no
273 development of tolerance to C&D agents was observed for the multigenera biofilms during the
274 course of the experiment. The *L. monocytogenes* biofilms, however, did develop increased

tolerance over time, as no significant reductions in CFU was observed during the C&D process after the coupons had been exposed to three daily cycles of cleaning followed by either disinfection with PAA or a QAC.

All strains were susceptible to the sanitation agents in suspension tests. To examine whether any of the strains included in the multigenera biofilms had a specific tolerance towards the employed C&D agents that could explain survival, bactericidal suspension tests were performed on each strain, using both QAC and PAA disinfection agents as well as the Alkalifoam cleaning agent. For all strains, the bacterial reductions were over 4 $\log_{10}$ units after exposure to recommended user concentrations of the QAC and PAA disinfectants for 5 min at 12°C (Table S3 in the Supplemental Material). Most strains also showed the same level of tolerance to the cleaning agent alone. The exceptions were the two *Corynebacterium* sp. strains and the *Micrococcus* sp. strain, which showed only between 10 and 100-fold reduction in CFUs upon treatment with the cleaning agent, and the two *Kocuria* sp. strains, *P. putida* strain MF6396, and the *Psychrobacter* sp. strain, which showed 3 $\log_{10}$ to 4 $\log_{10}$ reductions in CFU per ml upon treatment with the cleaning agent. These results indicate that all strains were susceptible to the C&D treatment when grown in suspension.

CLSM analysis showed that biofilms predominantly settle on the underside of the conveyor belt material. Confocal laser scanning microscopy (CLSM) was employed to examine the spatial organization of biofilms formed on the conveyor belt coupons. The three-dimensional image reconstructions shown in Fig. 6 and Fig. 7 were obtained by scans of several

pre-defined location patterns on each coupon (see Fig. 6D), and were selected from 174 acquired confocal Z-stack scans (see Table S4 in the Supplemental Material). In the majority of captured scans, relatively few sparse cells – attached singly or as small clusters – were observed on the coupon surface. However, a significant number of images showed the presence of large heterogeneous three-dimensional biofilms. These were also observed on some of the coupons examined immediately after cleaning and disinfection with QAC or PAA. In the multigenera biofilms, GFP expressing *L. monocytogenes* cells were often absent despite observations of significant numbers of background flora cells. When present, *L. monocytogenes* were spatially organized as single cells mixed in between the cells of the background flora strains. In some of the images, the biofilm also appeared to have a slightly layered structure, with *L. monocytogenes* cells found closer to the bottom layer of the biofilms (Fig. 7K). No separate *L. monocytogenes* monospecies microcolonies were observed on the coupons in which multigenera biofilms were grown.

The top face of the conveyor belt is coated with PVC, and is a matt antistatic surface (Fig. 6D). The underside of the conveyor belt is an urethane-impregnated woven polyester fabric. The photomicrograph in Fig. 7B, taken of the underside of a coupon, shows the linen weave pattern with single smooth warp threads and weft threads composed of bundles of smaller fibers. The difference between the flat top face and the heterogeneous topography of the rear face of the conveyor belt coupons can be seen in the overview images obtained by stitching together multiple CLSM scans – acquired across the length of the coupon from top to bottom – shown in Fig. 6E and Fig. 7D. Notably, for the rear side of the coupons, scans were only obtained for the

most elevated parts of the fabric, since the microscope was not able to focus in the areas constituting the «valleys» in the fabric surface. Most striking were images acquired for biofilms formed on weft threads composed of bundles of smaller fibers on the rear side of the conveyor belt coupons, as shown in Fig. 7E and F. Both the background flora and *L. monocytogenes* cells are predominantly found in the gap between these fibers. In the multigenera biofilms, mushroom shaped biofilm structures could be observed to protrude upwards from the cleft harboring bacterial cells (Fig. 7G).

Quantitative analysis of the biovolume of GFP expressing *L. monocytogenes* cells in the biofilms was performed by analysis of the green channel of the acquired CLSM image stacks (Table 3 and Fig. S5 in the Supplemental Material). The calculated biovolume of *L. monocytogenes* cells was higher prior to C&D than after treatment with QAC or PAA. Also, the results suggest that the total *L. monocytogenes* biovolume was higher in biofilms harvested on Day 7 compared to biofilms harvested on Day 4, both in multigenera biofilms and in *L. monocytogenes* single species biofilms. Finally, the analysis strongly indicates that significantly more *L. monocytogenes* cells were attached to the woven-structured underside of the conveyor belt than on the PVC-coated top surface. The strongest effect was seen for *L. monocytogenes* biofilms rinsed in H_2O daily from Days 4 to 7 and harvested on Day 7, in which 14 (SE±11) μm^3 and 2841 (SE±1439) μm^3 *L. monocytogenes* cells were found on the top and bottom faces of the conveyor belt coupons, respectively.

In summary, the microscopy showed that *L. monocytogenes* cells were spatially intermixed with background flora species in the multigenera biofilms. Furthermore, bacteria appeared to be

predominantly situated in the gaps between filament fibers on the underside of the conveyor belts.

DISCUSSION

The current study aimed to decipher growth and survival of *L. monocytogenes* on conveyor belts in food industry using conditions relatively realistic for those found in meat production environments. This included growing strains of *L. monocytogenes* in multigenera biofilms with strains from the background microbiota isolated in these environments. Initial investigation of the microbiota on conveyor belts after C&D in two RTE meat-processing plants resulted in isolation of a relatively small number of bacteria, but nevertheless, a high diversity was found between and also within samples (Table 1). A relatively diverse microbiota was therefore used in the initial biofilm experiments (Table 2). Similar to what has been found in other studies, *Pseudomonas* was relatively common after C&D (5-7, 30-32). *Enterobacteriaceae* has also been reported to be common in meat processing environments (5-7, 30, 31, 33, 34), but was absent in our study. Instead, microbiota of conveyor belts were dominated by bacteria less frequently reported in previous studies, such as *Acinetobacter*, *Microbacterium*, *Sphingomonas* and *Epilithonimonas* (Table 1). The composition of the microbiota is dependent on a number of factors such as the sanitation regime, the temperature and the humidity. Biofilm formation reflecting all these varying conditions would not be possible in *in vitro* laboratory studies. In this study, we chose to simulate conditions with high humidity and nutrient content at a temperature relevant for meat processing environments (12°C), and apply C&D cycles similar to those found in food industry.

358 The composition of the biofilms formed on conveyor belt coupons under these conditions was
359 largely stable – with a dominance of *Pseudomonas* and *Acinetobacter* strains – regardless of
360 whether four or 16 background strains were used as inocula, and regardless of whether
361 coupons had been treated with QAC, PAA, or rinsed in H_2O (Fig. 1A and B). Stable coexistence of
362 *Acinetobacter* and *Pseudomonas* strains in biofilms has been reported previously (35, 36). In the
363 current study, the composition of the biofilms shifted from an *Acinetobacter*-dominated biofilm
364 in the Day 4 samples to a *P. putida*-dominated biofilm in the Day 7 samples subjected to daily
365 C&D (Fig. 1A and B). This transition was not seen in biofilms instead subjected to daily rinse in
366 H_2O on Days 4 to 7 (Fig. 1B; column labelled H_2O). Furthermore, in suspension, *P. putida*
367 MF6396 had a higher tolerance towards the lethal effect of the chloralkali cleaning agent than
368 *A. johnsonii* and the other included *Pseudomonas* strains. This suggests that the dominance of
369 the *P. putida* strain in biofilms subjected to daily C&D could be a consequence of the C&D
370 treatments and tolerance of the *P. putida* strain towards the cleaning agent. However, the
371 relative levels of *Acinetobacter* decreased over time also in the planktonic competition
372 experiments performed in the current study (Fig. 1C) and in multigenera biofilm experiments
373 performed in a previous study, in which the effect of C&D was not assessed (37). Potentially,
374 interspecies interactions such as competition for limiting nutrient sources may also have
375 contributed to the observed transition in microbial composition between the Day 4 and Day 7
376 biofilms.

377 Specific bacteria may show enhanced survival in biofilms challenged by biocides by means of
378 interspecies interactions such as coaggregation and metabolic cross-feeding (27). Interactions

379 with other bacteria in biofilms may potentially explain the persistence of pathogens such as *L.*
380 *monocytogenes* in food production environments. In the current study, the proportion of *L.*
381 *monocytogenes* in the multigenera biofilms increased during the course of the experiment,
382 concomitant with the shift towards a *P. putida*-dominated biofilm. This is consistent with *L.*
383 *monocytogenes* specifically interacting with the *P. putida* strain. Interestingly, this specific strain
384 (MF6396) was isolated from a conveyor belt which was persistently contaminated with *L.*
385 *monocytogenes*, and from which the persistent ST8 strain *L. monocytogenes* MF5377 was
386 isolated (see Table 1) (38). It is therefore likely that MF6396 and MF5377 may have originated
387 from the same microhabitat in the meat production plant. Examination of biofilms using CLSM
388 in the current study showed that cells of *L. monocytogenes* were found intermixed with
389 background flora cells, with no spatially segregated *L. monocytogenes* microcolonies observed
390 within the multigenera biofilms (Fig. 6 and Fig. 7). Such spatial distribution patterns in
391 multispecies biofilms are indicative of interspecies coaggregation and cooperation (39, 40),
392 further suggesting that *L. monocytogenes* cells may directly interact with one or more of the
393 other species found in the biofilm. Previous studies have shown that co-culture of *L.*
394 *monocytogenes* and resident apathogenic bacteria from food production environments have
395 resulted in both positive and negative effects on the biomass of *L. monocytogenes* (29, 41).
396 Potential specific interactions between the individual strains examined in the current study are
397 subject to further examination in our laboratory.

398 The observation that certain subtypes of *L. monocytogenes* are more likely than others to
399 persist in food processing environments has prompted several investigators to examine whether

400 genetic determinants or various phenotypic traits could be associated with this ability. One of
401 the aims of the current study was to examine whether this perceived persistence may be linked
402 to strain-specific differences in the ability of *L. monocytogenes* to interact with the resident
403 microflora in biofilms. Few studies have addressed this point specifically, although in a recent
404 study, Overney et al. (42) found that two reference strains of *L. monocytogenes* (EGD-e and
405 LO28) did not differ in survival rate when they were grown in dual culture biofilms with a *P.*
406 *fluorescens* strain, when biofilms were subject to daily cycles of C&D and desiccation. A similar
407 result was obtained in the current study, where seven *L. monocytogenes* strains – four of which
408 belonged to subtypes linked to persistent contaminations in food production facilities – were
409 shown to be equally capable of growth and survival in biofilms exposed to C&D (Fig. 2). This
410 result was obtained both with monospecies and multigenera biofilms, and is consistent with the
411 growing consensus that individual genetic traits linked to specific subtypes do not account for
412 the existence of persistent subtypes of *L. monocytogenes* (13, 14).

413 It is widely acknowledged that the efficacy of C&D agents is lower for biofilms than for bacteria
414 growing in planktonic culture (3, 4, 27). A high level of tolerance to C&D was also observed for
415 biofilms in the current study, with less than 1 $\log_{10}$ reductions in total CFUs per coupon obtained
416 across treatments, when the C&D agents were applied at the concentrations recommended by
417 the manufacturers (Fig. 5). A similar level of efficacy of C&D agents applied at recommended
418 user concentrations was seen in a study by Pan et al. (43), where *L. monocytogenes* biofilms –
419 grown on stainless steel or Teflon coupons and subjected to daily cycles of sanitation followed
420 by starvation and incubation in dilute culture medium – were followed over a period of three

421 weeks. In their study, treatments of biofilms with minimum recommended user concentrations
422 of peroxide, QAC or chloride disinfection resulted in less than 0.3 $\log_{10}$ CFU cm^{-2} after the first
423 week of their simulated food processing regimen. However, not all studies find the efficacy of
424 C&D agents against biofilms to be this low – in some studies the disinfection agents have to be
425 diluted below recommended user concentrations in order to maintain enough cells above the
426 detection threshold after disinfection of biofilm coupons (42). Also, previous observations of
427 PAA being more effective against *L. monocytogenes* biofilms than QAC (44) was not supported
428 by the results obtained in the current study. In any case, the explanation for the low efficacy of
429 C&D seen in the current study cannot be attributed to the greater tolerance towards biocides
430 commonly observed for multispecies biofilms compared with their single species counterparts
431 (4, 23, 26), because the opposite was actually observed: *L. monocytogenes* biofilms were shown
432 to become more tolerant to daily C&D than the multigenera biofilms (Fig. 5). Since no significant
433 difference in survival of bacteria was observed between treatments with QAC or PAA
434 disinfectants, the low efficacy of C&D was furthermore not likely to be a result of specific
435 resistance mechanisms such as the presence of efflux pumps conferring resistance towards
436 chemical agents. This is supported by the observation that no selection between different *L.*
437 *monocytogenes* strains was seen despite two of the strains possessing the *qacH* gene encoding
438 an efflux pump conferring increased tolerance to low concentrations of QAC compounds (Table
439 2) (45).

440 The explanation for the low efficacy of the C&D treatment could instead, at least partly, be
441 ascribed to features of the coupon material on which biofilms were grown. Within food

442 processing plants, conveyor belts have been shown to be favorable to contaminations with *L.*
443 *monocytogenes* that are difficult to remove (9, 46). Furthermore, cracks or scratches in the
444 surfaces of materials used in food industry been shown to support development of *L.*
445 *monocytogenes* biofilms deeply rooted in microscopic sutures and ridges (47). The underside of
446 the conveyor belt used as the surface for biofilm growth in the current study had a woven
447 surface with filament fiber threads. When coupons were viewed using CLSM, bacteria could be
448 seen to shelter in the clefts between these fibers (Fig. 7), and quantitative biovolume analysis
449 furthermore suggested that significantly more *L. monocytogenes* cells were attached to the
450 underside of the conveyor belt than on the smooth top coating (Table 3). Bacteria could also be
451 expected to find harborage sites on the cut edges of the conveyor belt coupons, which –
452 although likely to be sealed to prevent penetration of soiling and bacteria when conveyors are
453 initially installed in food production plants – could be said to model situations where worn or
454 frayed conveyors are employed in a production facility.

455 The observed increase in tolerance to C&D by *L. monocytogenes* biofilms over time both for the
456 QAC and PAA disinfectants (Fig. 5) concurs with results obtained in the study by Pan et al. (43),
457 in which *L. monocytogenes* appeared to develop similar levels of biofilm-specific resistance to
458 disinfection with peroxide, QAC and chloride during the course of the experiment. This indicates
459 that a broad-spectrum mechanism, probably related to the biofilm mode of growth, was
460 responsible for the increased tolerance seen in both studies. This increase may potentially also
461 be linked to attributes of the coupon surface on which biofilms were grown. When biofilms
462 were examined using CLSM in the current study, larger *L. monocytogenes* biofilm aggregates

were always seen confined to the clefts and surface structures on the underside of the conveyor belt material, while the multigenera biofilms were regularly observed to protrude outwards from the crevices in which they were rooted. Conceivably, spatial growth patterns and/or a relatively modest growth rate could account for *L. monocytogenes* biofilms not extending beyond the shelter of the crevices the during the 24 h separating two cycles of C&D, thereby resulting in the observed lower reduction in *L. monocytogenes* numbers upon C&D on Day 7 compared to on Day 4 (Fig. 5).

In summary, the results from the present study showed that *L. monocytogenes* can grow and survive in multigenera biofilms formed from bacteria belonging to the background microbiota isolated in meat industry environments, even after several rounds of C&D. Furthermore, the results suggest that regular C&D agents used in food industry fail at removing biofilms from heterogeneous surfaces harboring cracks or crevices. Although the underside of a conveyor belt is not intended to be in direct contact with food, it may confer harborage sites from which bacteria can shelter and cross-contaminate food-contact surfaces during processing. Further research into more efficient methods for removal of biofilms and a greater focus on hygienic design of food processing equipment is warranted.

MATERIALS AND METHODS

Isolation of bacteria from conveyor belts in meat processing plants. Two plants processing RTE meats were visited. Samples from a total of nine conveyors – six from Plant A and three from Plant B – were taken after C&D, before start of production. The daily sanitation

483 included a chloralkali agent for cleaning followed by disinfections using QAC in Plant A and PAA
484 in Plant B. In addition, the conveyors in Plant B were disinfected with 70% ethanol several times
485 during the production day, between processing of different products and before breaks. An area
486 of approximately 900 cm^2 was sampled with neutralizing sampling cloths (Sodibox, Nevez,
487 France). The cloths were stored at 4°C and analyzed within 36 h. Ten ml peptone water (1 g l^{-1}
488 peptone [Oxoid], 0.85% NaCl, pH 7.2) was added to the plastic bag containing the cloth, and
489 after 30 seconds treatment in a Stomacher, 1 ml samples were plated to blood agar directly and
490 after dilution in order to obtain single well separated colonies for identification. The agar plates
491 were incubated at 20°C for 5 days. Up to 20 colonies were picked at random, restreaked for
492 purification and subjected to 16S rRNA sequencing (V3-V4 region) for identification using the
493 universal 16S rRNA primers tcctacgggaggcagcagt and ggactaccagggtatctaatcctgtt (48), as
494 previously described (37). The taxonomy of each strain was assigned by using the SeqMatch tool
495 of the Ribosomal Database Project (RDP), with database v.11.5 (https://rdp.cme.msu.edu).

496 **Selection criteria for background microbiota strains included in biofilm experiments.** A
497 total of 16 strains isolated from conveyor belts in meat processing Plants A and B were selected
498 for inclusion in multigenera biofilm experiments (Table 2). Of these, 14 isolates represented the
499 nine most frequently found genera after C&D. All these genera were among the dominating
500 (>20% of the colonies) in at least one sample. More than one isolate were chosen from genera
501 with diversity in the 16S rRNA amplicon sequences. In addition, *Kocuria* isolates were included
502 because *Kocuria* spp. has been reported to promote biofilm formation of *L. monocytogenes* in

503 an earlier study (29). All strains included in the experiments had unique 16S rRNA amplicon
504 sequences, enabling their differentiation by 16S rRNA sequencing.

505 **Selection criteria for *L. monocytogenes* strains.** Seven *L. monocytogenes* strains from three
506 different meat processing facilities [Plant A and B and a third plant; Plant C; corresponding to
507 plants M2, M4, and M1, respectively, from Møretrø et al. (22)] were selected for inclusion in
508 experiments in the current study (Table 2). These strains had been collected as part of two
509 research projects where nine Norwegian food processing plants were sampled, resulting in
510 isolation of a total of 680 *L. monocytogenes* strains subsequently typed using multiple locus
511 variable number tandem-repeats analysis (MLVA) (22). Of the seven strains used in the current
512 study, four were from MLVA profiles that were identified as persistent in the said projects using
513 the statistical approach described in Malley et al. (49) (results to be published separately), and
514 which were detected after C&D in more than one of the nine sampled Norwegian facilities. The
515 four included persistent strains were furthermore selected from individual strains isolated after
516 C&D at sampling points in which the same MLVA genotype had been found on several
517 occasions. Three *L. monocytogenes* strains were selected from so-called sporadic MLVA profiles.
518 These strains were selected based on the criteria that they should be isolated during
519 production, and that their MLVA profiles were not commonly found after C&D. All seven
520 selected strains had different *dapE* alleles, enabling their differentiation by sequencing the *dapE*
521 MLST allele (50). Alleles and sequence types for MLST were compared with those available in
522 the Institute Pasteur's *L. monocytogenes* MLST database
523 (http://bigsdb.web.pasteur.fr/listeria/listeria.html).

524 **Whole genome sequencing.** DNA isolation, whole genome sequencing and *de novo* genome
525 assembly was performed essentially as previously described (38), with 300 bp paired-end
526 sequencing on a MiSeq instrument (Illumina), except that genome assembly was performed
527 with v3.10.0 of SPAdes (51) and inclusion of six k-mer sizes (21,33,55,77,99,127). Contigs with
528 size <500 bp and with coverage <35 were removed from the assemblies. The sequences were
529 annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) server
530 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

531 **Sequence search for known biofilm genes.** The *Pseudomonas* and *Acinetobacter* genome
532 assemblies from the current study were analyzed for the presence of genes known to be
533 involved in biofilm formation in these genera (52-54) using BLAST+ v2.2.30 (55). The following
534 genes were used as queries in the analysis: *pslA-R* (PA2231-PA2246), *pelA-B* (PA3064-PA3058),
535 the *alg* operon (PA3540-3551), and *cdrA* (PA4625) from *P. aeruginosa* PAO1 (Accession
536 AE004091), genes *wssA-J* from *P. fluorescens* SBW25 (Accession AY074776), genes *lapA-G*
537 (PP018-PP0164), *lapF* (PP0806), the *bcs* operon (PP2629-PP2638), *peaA-I* (PP3133-P3141), and
538 the *peb* locus (PP1795-PP1788) from *P. putida* K4220 (Accession AE015451), the *csuA-E* genes
539 encoding the pilus usher-chaperone assembly system from *A. baumannii* 19606 (Accession
540 AY241696), *pgaA-D* (A1S_2160-2 and A1S_3792) from *A. baumannii* ATCC 17978 (Accession
541 CP000521), and the gene encoding Bap from *A. baumannii* 307-0294 (Accession EU117203).
542 Genome comparisons were performed using Mauve (56).

543 **Phylogenetic analysis.** The sequences of single genes or whole genomes from reference
544 strains used in phylogenetic analyses were downloaded from Genbank, and their accession
545 numbers are listed in Table S5 in the Supplemental Material. *Acinetobacter* strains were typed
546 *in silico* using the MLST scheme described by Diancourt et al. (57), while *Pseudomonas* strains
547 were analyzed using the MLSA scheme described by Mulet et al. (58). The concatenated
548 sequences of the seven MLST alleles (for *Acinetobacter*) or the four MLSA alleles (for
549 *Pseudomonas*) were aligned using CLCMain Workbench 7 (CLCbio). Phylogenetic trees were
550 then inferred from the alignments in MEGA7 (59) using the Neighbor-Joining method. The
551 evolutionary distances were computed using the Jukes-Cantor method and bootstrap
552 confidence values were generated using 1000 replicates.

553 **C&D agents.** C&D agents used in the current study were selected to represent products with
554 concentrations of active ingredients typical of industrial formulations. The industrial chlorinated
555 alkaline cleaning agent ISS Alkalifoam 27 (Ecolab, Norway), referred to as «Alkalifoam»
556 throughout the text, was used at a 1% concentration, which is the minimum recommended user
557 concentration indicated by the manufacturer. At this concentration the solution contains
558 minimum 0.02% NaOH and 0.03% sodium hypochlorite. Two industrial disinfection agents were
559 used. One was Aco Hygiene Des QA (Aco Kjemi, Norway), which is a formulation based on
560 quaternary ammonium compounds, referred to as «QAC» throughout the text. The second was
561 Diverfoam active (Lilleborg, Norway), which is based on peracetic acid, and referred to as «PAA»
562 throughout the text. Both are used at the indicated minimum user concentrations, which was
563 1% for QAC and 1.5% for PAA. At these concentrations, the QAC solution contains minimum

564 0.05% benzalkonium chloride while the PAA solution contains minimum 0.02% peracetic acid,
565 0.05% acetic acid, and 0.15% hydrogen peroxide.

566 **Growth conditions in planktonic culture.** Bacteria were grown in brain heart infusion (BHI)
567 broth (Oxoid) throughout all experiments. Overnight cultures and precultures were grown in 5
568 ml volumes in culture tubes and 50 ml Nunc-tubes, respectively, with shaking at 30°C, except for
569 *Sphingomonas* sp. MF4632, which was grown at 20°C. All biofilm and growth experiments were
570 carried out at 12°C. For plating, RAPID'L.mono (RLM) agar (Bio-Rad) and BHI agar (Oxoid) plates
571 were used.

572 For generation of growth curves for single strains, overnight cultures were diluted to
573 approximately 10^5 CFU ml^{-1} and inoculated in volumes of 250 µl in 100-well polystyrene
574 microwell plates (Oy Growth Curves Ab Ltd). The plates were incubated for 7 days at 12°C in a
575 Bioscreen C instrument (MTX Lab Systems Inc), with continuous shaking and recording of
576 OD_{600nm} every hour. Blank wells contained BHI broth only, and values for blanks were subtracted
577 from sample values to obtain actual absorbance measurements. Triplicate wells were used for
578 each sample and each strain was tested three or four times.

579 For the planktonic competition experiment, overnight cultures were mixed in roughly equal CFU
580 numbers in an inoculum diluted to a final total concentration of 10^5 CFU ml^{-1}. Fifty ml culture
581 volumes were incubated in 500 ml baffled Erlenmeyer bottles at 12°C with shaking at 200 rpm.
582 Every 24 h, samples were withdrawn and plated to determine CFU count, and cells were

583 pelleted by centrifugation and stored at -20°C for use in amplicon sequencing analysis (see
584 below).

585 **Construction of GFP-labelled *L. monocytogenes*.** *L. monocytogenes* strains were
586 transformed with plasmid pNF8, from which the green fluorescent protein (GFP) is constitutively
587 expressed (60). The pNF8 plasmid was a kind gift from Hanne Ingmer at the University of
588 Copenhagen. Transformation was performed using the procedure described by Monk et al. (61).
589 Erythromycin at a concentration of 10 µg ml^{-1} was used for selection of pNF8. The identity of all
590 strains after transformation was confirmed by PCR amplification and sequencing the *dapE* MLST
591 allele (50) using primers gttttcccagtcacgacgttgtacgactaatgggcatgaagaacaag and
592 ttgtgagcggataacaatttcatcgaactatgggcatttttacc for PCR (overhangs underlined) and primers
593 gttttcccagtcacgacgttgta and ttgtgagcggataacaatttc for sequencing.

594 **Biofilm experiments with C&D.** Precultures of each strain were inoculated from glycerol
595 stocks, prepared from exponential phase cultures and maintained at -80°C, grown separately to
596 logarithmic phase, and mixed in roughly equal CFU numbers in an inoculum diluted to a final
597 total concentration of ~10^6 CFU ml^{-1}. The bacterial suspensions were inoculated in 24-well
598 plates containing coupons of food grade PVC conveyor belt material (Forbo-Siegling Transilon; E
599 8/2 U0/V5 MT white FDA) cut to 1.0 cm × 1.5 cm, autoclaved, and placed vertically in each well.
600 One ml inoculum was added to each well so that wells were half-filled with culture broth,
601 resulting in the air/liquid interface crossing the length of the coupon (see Fig. 6D). The plates

602 were incubated at 12°C with gentle orbital shaking, and the culture medium was refreshed on
603 Day 3.

604 Control coupons not subjected to C&D were harvested after four days of biofilm development.
605 Sets of coupons subjected to C&D (see below) on Day 4 were either harvested after treatment
606 or – for coupons to be harvested on Day 7 – placed in a new 24-well tray containing 1 ml BHI in
607 each well and incubated as before for 24 h. The cycles of C&D followed by incubation in BHI was
608 repeated on Days 5 and 6. On Day 7, sets of coupons treated with either QAC or PAA on Days 4
609 to 6 were harvested prior to and after C&D treatment. Coupons sampled prior to C&D (both on
610 Days 4 and 7) were rinsed three times in ~10ml H_2O (in 15 ml Falcon tubes) to remove non-
611 adherent bacteria before harvest. Control coupons subjected to rinsing in H_2O instead of
612 treatment with C&D agents on Days 4 to 7 were included in selected experiments.

613 Treatment with C&D agents was performed as follows: C&D agents were applied as foam (as
614 intended by the manufacturers), produced in foam pump bottles (Sunvita, Norway). Each
615 coupon was rinsed three times in ~10 ml H_2O (in 15 ml Falcon tubes) and placed vertically in
616 wells of a clean 24-well tray. The wells were filled with 1% Alkalifoam, coupons were incubated
617 5 minutes, rinsed as before in H_2O, and placed in a second clean 24-well plate. The wells were
618 then filled with 1% QAC or 1.5% PAA, coupons were incubated 5 minutes, and finally rinsed as
619 before in H_2O. The average weight of foam applied to each well was ~350 mg.

620 Cells attached to coupons were harvested as follows: Each coupon was transferred to a glass
621 tube containing 4.5 ml peptone water and 2 g glass beads of diameter ~2 mm (Assistant, No.

622 1401/2). Tubes were then vortexed for 30 seconds and sonicated for 10 minutes (Branson 3510
623 ultrasonic cleaner) to dislodge attached cells and disperse cell aggregates. After withdrawing
624 45.5 µl or 500 µl for plating dilutions on agar plates (to determine total and *L. monocytogenes*
625 CFU count per coupon), the remaining cells were pelleted by centrifugation (16000×g for 5 min)
626 and stored at -20°C. The identity of single *L. monocytogenes* colonies from dilutions plated after
627 harvesting coupons subjected to sanitation on Day 7 was determined by PCR amplification and
628 sequencing the *dapE* MLST allele (50) as described above.

629 Biofilms analyzed using CLSM were grown and subjected to rinsing or C&D as described above,
630 with the following exceptions: For *L. monocytogenes*, the strains labelled with GFP were used,
631 and overnight cultures for these were grown in the presence of 10 µg ml^{-1} erythromycin. The
632 biofilm inoculum was prepared from overnight cultures diluted to an OD_{600nm} of 0.01. These
633 were mixed so that the inoculum contained 12.5% v/v of each of the four background
634 microbiota strains *Acinetobacter* MF4640 and *Pseudomonas* strains MF4836, MF6394 and
635 MF6396, and 50% of a mixture of equal amounts of the seven *L. monocytogenes* strains (Table
636 2). Biofilms were grown under static conditions. Rinsing of coupons in H_2O before and after
637 treatment with C&D agents was performed three times in 2.5 ml volumes of H_2O in 24-well
638 plates. After coupons were either subjected to C&D or rinsed in H_2O (to remove non-adherent
639 bacteria from control coupons), coupons were left in BHI until imaging the same day.

640 **DNA isolation and amplicon sequencing.** For purification of genomic DNA for amplicon
641 sequencing analysis, cells were lysed using Lysing Matrix B and a FastPrep-24 instrument (both
642 MP Biomedicals) and DNA isolated using either the PowerSoil-htp 96 Well Soil DNA Isolation Kit

643 (MoBio) (biofilm experiments #1 to #3) or the DNeasy Blood and Tissue Kit (Qiagen) (biofilm
644 experiments #4 and #5 and planktonic competition experiments). Libraries for amplicon
645 sequencing to analyze microbial composition were prepared following the 16S Metagenomic
646 Sequencing Library Preparation protocol from Illumina (62). Briefly, amplicon PCR was
647 performed with primers targeting either the V3-V4 region of the 16S rRNA gene or the *dapE*
648 gene of *L. monocytogenes*, followed by an index PCR performed using the Nextera XT index kit
649 (Illumina). The primers used to amplify the 16S rRNA gene were
650 tcgtcggcagcgtcagatgtgtataagagacagcctacgggnggcwgcag and
651 gtctcgtgggctcggagatgtgtataagagacaggactachvgggtatctaatcc, and those used to amplify *dapE*
652 were tcgtcggcagcgtcagatgtgtataagagacagcgactaatgggcatgaagaacaag and
653 gtctcgtgggctcggagatgtgtataagagacagcatcgaactatgggcatttttacc (overhangs underlined). PCR
654 products were purified using the AMPure XP system (Agencourt) after each PCR and after
655 pooling. Purified indexed PCR products and the pooled sample were quantified using the Quant-
656 iT Picogreen dsDNA kit (Invitrogen). The library was spiked with 10% PhiX control and
657 sequenced using MiSeq v3 reagents using paired 300 bp reads on a MiSeq instrument (Illumina).

658 **Metagenomic Analysis using Qiime software.** Demultiplexed raw reads from the MiSeq run
659 were processed with the Qiime software package (Quantitative Insights Into Microbial Ecology)
660 v1.9.1 (63): After paired end reads were joined, they were quality filtered on q20. Then, samples
661 amplified with *dapE* primers were assigned to their respective *dapE* allele using a closed
662 reference OTU picking protocol against a custom reference file containing the *dapE* allele
663 sequences of the seven *L. monocytogenes* strains (*dapE*-alleles numbers 4, 6, 7, 8, 9, 18, 20, and

21 as listed at the Institute Pasteur's *L. monocytogenes* MLST database at http://bigsdb.web.pasteur.fr/listeria/listeria.html). The OTU picking script was run with default parameters except that the sequence similarity threshold was set to 1. For samples amplified with 16S rRNA primers, samples were analyzed using an open reference OTU picking protocol, in which reads were first matched against a custom reference file containing the 16S rRNA allele sequences of *L. monocytogenes* plus the 16 background flora strains included in the experiments. The 16S rRNA reference file is included as Table S6 in the Supplemental Material.

Bactericidal suspension test. Overnight cultures were diluted to approximately 10^8 CFU ml^{-1} in peptone water and 1 ml of the diluted culture was added directly to 9 ml of H_2O (control) or user concentrations of Alkalifoam (1%), QAC (1%) or PAA (1.5%) resulting in a final cell concentration of approximately 10^7 CFU ml^{-1}. After 5 min, 0.5 ml of the solution was transferred to Dey Engley (D/E) neutralizing broth (Difco) and dilutions were plated on BHI agar plates. The tests were performed with all solutions at 12°C. The experiment was performed three to four times for each strain.

Confocal laser scanning microscopy (CLSM). Surface-associated bacteria on conveyor belt coupons were stained with the cell-permeant Syto 61 red fluorescent nucleic acid strain (Life Technologies), diluted to 5 mM in DMSO and used at a 1:2000 dilution. *L. monocytogenes* were pinpointed in the complex biofilm through specific emission of their green GFP expression. Images were acquired using a Leica SP8 confocal laser scanning microscope (Leica Microsystems) at the MIMA2 microscopy platform (www6.jouy.inra.fr/mima2). Images were

obtained using a HC PL APO 63× long distance water objective with a numerical aperture of NA=1.2. The GFP green emitted fluorescence signal from *L. monocytogenes* cells was collected on a hybrid detector in the range 500-550 nm after excitation at 488 nm with an Argon laser set at 20% of its maximal intensity. The red fluorescence emitted by the bacteria labeled with Syto 61 was collected on a photomultiplier in the range 645-675 nm after excitation with a 633 nm HeNe laser. In order to contrast the surface topography, reflected signal from the 633 nm HeNe laser was collected. Samples were scanned at 600 Hz every micron with 246×246 µm images in order to acquire multi-color 3D stacks.

Representative CLSM images from each coupon were acquired by scanning Z-stacks on different locations of the sample with a fixed pattern on the coupon surface. In most cases, three or five stacks were taken from both the top face and underside of the conveyor belt coupon, at even intervals following a line from the bottom to the top of the coupon, as marked with crosses in the photograph of the coupon in Fig. 6D. The number of obtained Z-stack CLSM scans for the different samples and treatments is summarized in Table S4 in the Supplemental Material. On a few coupons, a larger view of the coupon was obtained thanks to a mosaic 3D meta-image, obtained using a motorized stage that automatically moves the sample between scans and tiles the adjacent fields. All CLSM stacks were processed using IMARIS (Bitplane) to projection images of the biofilms. Quantitative analysis of each Z-stack to quantify the green biovolume corresponding to *L. monocytogenes* subpopulation of the biofilm was performed using the ICY image analysis software (64) using a homemade script previously described, with some adaptations (65). The biomass software algorithm did not reliably score low cell numbers

correctly, therefore the biovolume of scans where ≤5 cells were observed in the green channel of the image projection was set to 0, when the scored biovolume was above a threshold of 800 μm^3. The biovolume of the red channel was not extracted since red fluorescent background interfered with the Syto 61 specific signal**.**

Accession numbers. This Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank under the accession numbers MVOJ00000000, MVOK00000000, MVOL00000000, MVOM00000000, and MVON00000000. The versions described in this paper are versions XXXX01000000. The raw reads are available from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under accession numbers SRR5273317, SRR5273318, SRR5273319, SRR5273320, and SRR5273321.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found in the file:

«**Supplemental Material**», PDF file, 1.2 MB

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925 TABLES

926 Table 1: Microbiota found on conveyor belts in meat processing plants

	Number of colonies of each genus isolated from each conveyor belt									**Total number of colonies of each genus:**
	Plant A						Plant B			
	1	**2**	**3**	**4**	**5**	**6**[b]	**1**	**2**	**3**	
Pseudomonas	7[a]	5	24[a]			1[a]				**37**
Acinetobacter	1	6						6[a]		**13**
Microbacterium						10[a]	1			**11**
Sphingomonas						1	10[a]			**11**
Epilithonimonas						8[a]				**8**
Micrococcus				4		1[a]			1	**6**
Psychrobacter	4							2[a]		**6**
Rhodococcus						1	4[a]			**5**
Corynebacterium								2[a]	2	**4**
Brevundomonas						1	2		1	**4**
Vagococcus	3									**3**
Erysipelothrix	2									**2**
Kocuria						1[a]		1[a]		**2**
Chryseobacterium							1			**1**
Exigubacterium					1					**1**
Leucobacter							1			**1**
Lysinibacillus	1									**1**
Moraxella					1					**1**
Paenibacillus		1								**1**
Rhothia									1	**1**
Roseomonas						1				**1**
Variovorax							1			**1**
Total number of colonies isolated from each conveyor belt:	**18**	**12**	**24**	**4**	**2**	**25**	**20**	**11**	**5**	SUM: 121

[a] *Sample with isolates used in biofilm experiments in the current study.*

[b] *L. monocytogenes was isolated from this conveyor belt on several other occasions (MF5377).*

927

928 Table 2: Bacterial strains used in biofilm experiments

Background microbiota from conveyor belts		
Strain	**Plant[a]**	**Bacterial species or genera**
MF4640	B	*Acinetobacter johnsonii*
MF4642	B	*Acinetobacter johnsonii*
MF4643	B	*Corynebacterium sputi*
MF4645	B	*Corynebacterium* sp.
MF6392	A	*Epilithonimonas* sp.
MF4644	B	*Kocuria* sp.
MF6395	A	*Kocuria rhizophila*
MF4634	B	*Microbacterium* sp.
MF6393	A	*Micrococcus* sp.
MF6396	A	*Pseudomonas* sp. (*P. putida* group)
MF6394	A	*Pseudomonas* sp. (*P. fluorescens* subgroup)
MF4836	A	*Pseudomonas* sp. (*P. mandelii* subgroup)
MF4641	B	*Psychrobacter* sp.
MF4633	B	*Rhodococcus erythropolis*
MF4637	B	*Rhodococcus fascians*
MF4632	B	*Sphingomonas* sp.
***L. monocytogenes* strains from meat processing environments[b]**		
Strain	**Plant[a]**	**MLST sequence type, MLVA profile[c]**
MF4536	C	ST9, MLVA 6-11-15-18-6 (persistent, *qacH* positive)
MF5376	A	ST7, MLVA 7-7-10-10-6 (persistent)
MF5634	B	ST121, MLVA 6-7-14-10-6 (persistent, *qacH* positive)
MF5377	A	ST8, MLVA 6-9-18-16-6 (persistent)
MF4565	C	ST18, MLVA 8-8-17-21-6 (sporadic)
MF5630	C	ST19, MLVA 6-9-18-10-6 (sporadic)
MF5378	A	ST394, MLVA 6-9-19-10-6 (sporadic)

[a] *Plants A, B, and C correspond to plants M2, M4, and M1, respectively, described in Møretrø et al. (22).*

[b] *L. monocytogenes strains were isolated in Møretrø et al. (22).*

[c] *Listed MLVA profiles correspond to variable number tandem repeat (VNTR) loci LMV6-LMV1-LMV2-LMV7-LMV9 described in Lindstedt et al. (66). Persistent or sporadic MLVA profiles are determined using the criteria described in the Materials and Methods section.*

929

930 Table 3: Biovolume of *L. monocytogenes* on conveyor belt coupons[a]

		Before C&D		After C&D	
Front of coupon		H_2O	QAC	QAC	PAA
Multigenera biofilms	Day 4	149 (±32)	NA	39 (±23)	0.06 (±0.03)
	Day 7	35 (±22)	1.3 (±1.2)	0.67 (±0.61)	NT
L. monocytogenes biofilms	Day 4	3.4 (±1.1)	NA	0.18 (±0.11)	0.1 (±0.08)
	Day 7	14 (±11)	NT	NT	NT
Back of coupon					
Multigenera biofilms	Day 4	331 (±141)	NA	11 (±5)	0 (±0)
	Day 7	62 (±14)	68 (±66)	0.1 (±0.06)	NT
L. monocytogenes biofilms	Day 4	172 (±73)	NA	0.61 (±0.61)	NT
	Day 7	2841 (±1439)	11 (±3,6)	6.3 (±3.9)	NT

NA: not applicable

NT: not tested

[a] Values are obtained from CLSM Z-scans using the ICY image analysis software (64) and are given as averages of the total volume (μm^3) per scan +/- the standard error of the mean for each tested combination. The number of coupons tested for each condition is given in Table S4 in the Supplemental Material.

931

FIGURE LEGENDS

FIG 1 Development of the microbiota in multigenera biofilms on conveyor belt coupons (A, B) and planktonic cultures (C) at 12°C. Inocula were composed of *L. monocytogenes* plus either 16 (A, C) or four (B) background microbiota strains. The frequencies of different bacterial strains were determined by 16S rRNA amplicon sequencing. (A, B) Biofilms were allowed to develop for four days before being subjected to daily cleaning with Alkalifoam and treatment with either a QAC or PAA disinfection agent or (B) a daily rinse in H_2O. Coupons were harvested either before or after C&D on the day of harvest, as indicated. (C) Development of microbiota in planktonic cultures, grown with shaking in Erlenmeyer flasks for a total of 72 h. Presented results are averages from three (A, C) or two (B) independent experiments. Results for individual experiments are shown in Fig. S3 in the Supplemental Material.

FIG 2 Competition between *L. monocytogenes* strains during biofilm (A) and planktonic growth (B) in BHI at 12°C. Inoculated suspensions were composed of equal amounts of seven different *L. monocytogenes* strains, grown either together with 16 background microbiota strains (multigenera biofilm/culture) or alone (*L. monocytogenes* biofilm/culture). (A) Frequencies of *L. monocytogenes* strains surviving in biofilms on conveyor belt coupons. Biofilms were allowed to develop for four days and then subjected to daily cleaning with Alkalifoam and disinfection with either a QAC- or PAA-based disinfectant on four consecutive days before harvest. The identity of single colonies of *L. monocytogenes* was determined by Sanger sequencing of the *dapE* allele. Results are pooled from three replicate experiments in which ten single *L. monocytogenes* colonies were identified from each of the four conditions analyzed; 120 colonies in total. (B)

953 Time-course experiment showing the relative abundance of *L. monocytogenes* strains in
954 planktonic cultures, grown with shaking in Erlenmeyer flasks for a total of 72 h. The frequencies
955 of different *L. monocytogenes* strains was determined by *dapE* amplicon sequencing. Presented
956 results are averages from three independent experiments. Results for individual experiments
957 are shown in Fig. S4 in the Supplemental Material.

958 **FIG 3** Growth of the 16 background microbiota strains and the seven *L. monocytogenes* strains
959 in BHI medium at 12°C. The experiment was performed in a Bioscreen C instrument, with
960 measurement of the absorbance at 600nm once every hour. Results shown are averages of
961 triplicate wells in one representative experiment out of three experiments performed. Strains
962 included in the biofilm experiments with four background microbiota strains (Fig. 1B) are
963 represented by solid colored lines. Dashed lines represent selected strains that also show good
964 growth properties. Grey lines represent the remaining eight strains.

965 **FIG 4** Total bacterial numbers in biofilms on conveyor belt coupons prior to C&D. Biofilms were
966 allowed to develop undisturbed until Day 4 before being subjected to rinsing in H_2O or to
967 cleaning with Alkalifoam and disinfection with either a QAC- or a PAA-based disinfection agent
968 on three consecutive days. The microbiota on coupons harvested on Day 7 had been allowed to
969 regrow for 24 h after the last disinfection step. The *L. monocytogenes* count in multigenera
970 biofilms was determined by plating on selective agar. Mean values of five replicates are shown,
971 except for the sample labelled H_2O, where the mean of two replicates is shown. Error bars show
972 standard error of the mean. Asterisks represent differences in *L. monocytogenes* CFU per

973 coupon relative to in multigenera biofilms harvested on Day 4 (*, $P = 0.08$; **, $P < 0.05$; two-
974 tailed paired Student *t* tests).

975 **FIG 5** Tolerance of biofilms to C&D regimes relevant in the food industry. The $\log_{10}$ reduction
976 upon C&D for multigenera biofilms and biofilms inoculated with seven *L. monocytogenes* strains
977 grown on conveyor belt coupons is shown. Biofilms were allowed to develop undisturbed until
978 Day 4 before being subjected to cleaning with Alkalifoam and disinfection with either a QAC- or
979 a PAA-based disinfection agent on four consecutive days. Calculated reductions are relative to
980 control coupons rinsed in H_2O only. The *L. monocytogenes* count in multigenera biofilms was
981 determined by plating on selective agar. Mean values of five experiments and standard error of
982 the mean are shown. Asterisks represent comparison of samples using the two-tailed paired
983 Student *t* tests (*, $P = 0.06$; **, $P < 0.05$).

984 **FIG 6** Biofilms on the top coating of conveyor belt material examined using CLSM. Green
985 represents GFP-expressing *L. monocytogenes* cells, while *Acinetobacter* and *Pseudomonas*
986 background flora strains are shown in red. CLSM images shown are Easy3D shadow projection
987 reconstructions obtained from the confocal Z-stack series using the IMARIS software. Coupons
988 with biofilm were imaged either on Day 4 (B) or on Day 7 (A, C, E) after initiation of biofilm
989 growth, and harvested after rinsing in H_2O (C, E) or after C&D with QAC (A, B). Panel (D) shows a
990 photograph of the front side of a conveyor belt coupon. The arrow indicates the approximate
991 location of the air-liquid interface during biofilm development, and crosses show the
992 approximate locations on each coupon where the CLMS image acquisitions were acquired
993 (when only three images were acquired, the locations indicated by the large crosses were used).

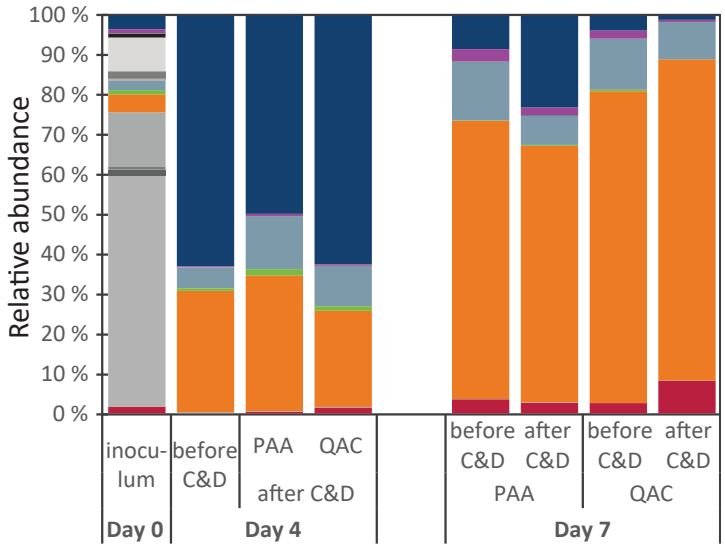
Panel (E) shows a to a mosaic 3D meta-image, obtained using a motorized stage that automatically moves the sample between scans and tiles the adjacent fields. The box drawn with dashed line in (D) approximately corresponds to the area of the coupon covered by the CSLM image in (E).

FIG 7 Biofilms formed on the underside of the conveyor belt examined using CLSM. *Pseudomonas* and *Acinetobacter* background flora strains (shown in red) and GFP-expressing *L. monocytogenes* strains (in green) were used as inoculum, except in panel (F), where biofilms were inoculated with *L. monocytogenes* strains only. (A, B) Photographs showing the underside of a clean conveyor belt coupon. The image in panel (B), photographed using an USB microscope lens, shows the weave pattern of the fabric with the smooth warp thread indicated by an arrow. Panels (C) to (H) show biofilms formed on the weft thread of the woven fabric, on coupons rinsed in H_2O on Days 4 to 7 (C, D, F), on a coupon rinsed in H_2O on Day 7 after treatment with QAC on Days 4 to 6 (E), and on coupons harvested on Day 4 for after treatment with PAA (G) or QAC (H). In panels (C, E, F), the smaller fibers constituting the weft thread is represented by the reflection signal (in grey). (C, D) Mosaic 3D meta-image, shown in section view mode (C) and Easy3D blend representation (D), with warp treads indicated by arrows. The boxes drawn with dashed lines in (A) and (B) show the size and location of the area of a coupon depicted by the CSLM images shown in panels (D) and (C), respectively. Panels (I) to (K) are different representations of the same scan, showing biofilm harvested on Day 4 after rinsing in H_2O, formed on the warp thread of the woven fabric. Images are shown as three-dimensional

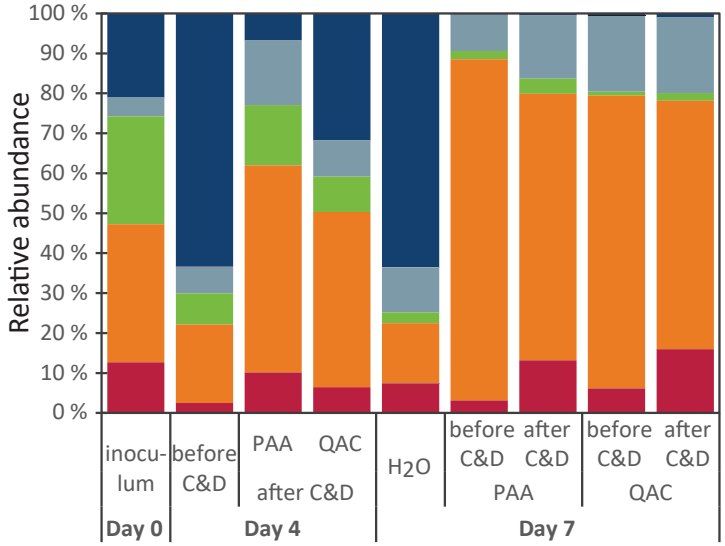
1014 (E, F, K) and Easy 3D (G, H, I, J) IMARIS representations. The scale is the same in the Easy3D

1015 blend images in panels (G) to (J). The red channel is not shown in panels (F) and (J).

A)



B)



C)