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Effects of the colonization sequence of *Listeria monocytogenes* and *Pseudomonas fluorescens* on survival of biofilm cells under food-related stresses and transfer to salmon

Xinyi Pang[a], Hyun-Gyun Yuk[b,*]

[a] *Food Science & Technology Programme, Department of Chemistry, National University of Singapore, Science Drive 4, 117543, Singapore*
[b] *Department of Food Science and Technology, Korea National University of Transportation, 61 Daehak-ro Jeungpyeong-gun, Chungbuk, 27909, Republic of Korea*

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ABSTRACT

This study evaluated how the colonization sequence of *Listeria monocytogenes* and *Pseudomonas fluorescens* affects biofilm formation and biofilm cell response to food-related stress (desiccation or disinfection) as well as the transferability of *L. monocytogenes* to salmon products. The results showed that the colonization sequence did not affect the population of dual species biofilms. Furthermore, survival number of *L. monocytogenes* was 0.8 log CFU/cm^2 higher when *P. fluorescens* was the first colonizer during desiccation or disinfectant treatment in comparison with dual-species biofilms with other colonization sequences. A lower transfer rate of *L. monocytogenes* biofilm cells from dual-species biofilms was observed as compared to single species biofilms. In particular, *L. monocytogenes* cells detached at a slower rate during transfer to 10 slices of salmon from dual-species biofilms first established by *P. fluorescens*. Confocal images revealed more exopolysaccharide production in dual-speciesbiofilms first established by *P. fluorescens* than in biofilms generated via other sequences. These results indicate that preexisting *P. fluorescens* biofilms on stainless steel can enhance resistance of *L. monocytogenes* to desiccation and disinfection, although this setup decreased the transfer rate of *L. monocytogenes* to salmon slices. Thus, this study highlights the risk of *L. monocytogenes* contamination in pre-formed *Pseudomonas* biofilms at salmon processing facilities.

1. Introduction

Listeria monocytogenes is a foodborne pathogen responsible for listeriosis, which poses significant risk to immunocompromised individuals, the elderly, fetuses, and newborns (Lomonaco et al., 2015). From 2009 to 2015 in the United States, although *L. monocytogenes* was responsible for 1% among 5760 foodborne disease outbreaks, it was the top pathogen, resulting in 52% of outbreak-associated deaths (Dewey-Mattia et al., 2018). In 2016, 2536 confirmed human cases of listeriosis were reported in Europe, of which 16.2% were fatal cases (EFSA, 2017). *L. monocytogenes* contamination mainly occurs during food processing rather than in raw food (Muhterem-Uyar et al., 2015; Vogel et al., 2001). Harborage sites on food industry premises such as irregularities of conveyor belts are preferentially colonized by *L. monocytogenes* (Fagerlund et al., 2017). The ability of *L. monocytogenes* to form biofilms on these food contact surfaces is possibly associated with its persistence in food processing environments (Nowak et al., 2017; Wang et al., 2015b).

As a possible mechanism of adaptation to various stresses, *L. monocytogenes* cells readily attach to food contact surfaces and form biofilms, which are shielded inside an extracellular polymeric substances (EPS) matrix (Donlan, 2002). Compared to their planktonic counterparts, biofilm cells are more resistant to food-related stresses such as antimicrobial agents and desiccation (Hansen and Vogel, 2011; Pan et al., 2006). An increasing number of studies have focused on interspecies interactions between *L. monocytogenes* and resident microorganisms in terms of population dynamics and biocide tolerance (Carpentier and Chassaing, 2004; Papaioannou et al., 2018; Rodríguez-López et al., 2015; Weiler et al., 2013). *Pseudomonas* spp. is a common species studied in co-culture biofilms because of its prevalence on food contact surfaces and its strong biofilm-forming ability (Langsrud et al., 2016). Moreover, some studies have revealed that *Pseudomonas* spp. may protect *L. monocytogenes* against biocides in dual-species biofilms formed by co-culture of bacterial suspension (Ibusquiza et al., 2012; Puga et al., 2016).

Biofilm formation is one of the intrinsic factors affecting bacterial

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[*] Corresponding author.
E-mail address: yukhg@ut.ac.kr (H.-G. Yuk).

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transfer via the surface-to-food contact route (Hansen and Vogel, 2011; Pérez-Rodríguez et al., 2008). Various studies evaluating the transfer of biofilm cells to meat products indicate that this transfer can be affected by the type of meat product, the culture medium for biofilm formation (Wang et al., 2015a), and biofilm properties, including structure, exopolysaccharide production and the attachment strengths of cells (Midelet and Carpentier, 2002; Lee and Wang, 2017). However, these studies were based on single-species biofilms, which may not accurately mimic the actual situations in food processing environments.

Little is known about mixed-species biofilms formed via different colonization sequences. Under real-life scenarios, food contact surfaces are likely conditioned by preexisting biofilms, which could change surface properties, with an impact on bacterial adhesion and subsequent biofilm formation (Van Houdt and Michiels, 2010). For instance, Wang et al. (2015c) reported that the colonization sequence of *Escherichia coli* O157:H7 and O111:H8 may affect biofilm population composition. Recently, Puga et al. (2018) uncovered enhanced attachment of *L. monocytogenes* to pre-formed *Pseudomonas fluorescens* biofilms compared to co-culture binary biofilms. Nevertheless, survival of *L. monocytogenes* in multispecies biofilms resulting from various colonization sequences under food-related stresses is still unknown. This information could improve our understanding of the behavior of *L. monocytogenes* in realistic biofilms on food industry premises and may point to new targets for biofilm eradication.

Accordingly, the objective of this study was to evaluate biofilm formation of *L. monocytogenes* and *P. fluorescens* on stainless steel (SS) surfaces in three colonization sequences and how these conditions affect bacterial survival under desiccation or disinfection stress. Moreover, the transfer of *L. monocytogenes* biofilm cells from SS surfaces to salmon products was investigated; this arrangement mimics the fate of *L. monocytogenes* in a food processing environment. Biofilm structure together with EPS production was examined to obtain a better understanding of the relation between biofilm matrix and biofilm cell survival.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. monocytogenes (SSA 151, serotype 1/2a), isolated from prepacked smoked salmon from a supermarket, was chosen as a strong biofilm former according to previously published results (Li et al., 2018). *P. fluorescens* (ATCC 13525) was purchased from the American Type Culture Collection (Manassas, VA, USA). Both strains were stored at −80 °C in separate cryoinstant vials with porous beads (DeltaLab, Barcelona, Spain). *L. monocytogenes* and *P. fluorescens* inocula were prepared by transferring one bead into a test tube containing 10 mL of sterile tryptic soy broth (TSB; Oxoid, Basingstoke, UK) and were incubated at 37 and 30 °C, respectively, for 24 h. After two consecutive transfers, the cultures were centrifuged at 5000 × *g* for 5 min at 4 °C. After that, the pellets were washed twice with phosphate-buffered saline (PBS; Vivantis Technologies Sdn. Bhd., Malaysia) and resuspended in 10 mL of PBS to obtain a working culture at a concentration of 10^8 CFU/mL.

2.2. Biofilm formation on SS surfaces

SS (314) coupons with the dimensions 2 × 1 × 0.2 cm were degreased by sonication in a detergent solution for 30 min and then in 70% (v/v) ethanol for 15 min. After a rinse in distilled water, the coupons were air-dried and autoclaved at 121 °C for 15 min.

For single-species biofilm formation, 200 µL of *L. monocytogenes* or *P. fluorescens* working culture was added into 20 mL of TSB, thereby yielding an initial concentration of 10^6 CFU/mL. Sterile SS coupons were then submerged into the bacterial suspension and incubated at 15 °C for 48 h. For dual-species biofilm formation, the SS coupons containing either *L. monocytogenes* or *P. fluorescens* single-species biofilms were washed with PBS and then inoculated with the second colonizer as follows. Under scenario 1 (colonization of preexisting *P. fluorescens* biofilms by *L. monocytogenes*, referred to as Pf-Lm biofilms), 20 mL of the *L. monocytogenes* inoculum was dispensed into a Petri dish containing SS coupons with *P. fluorescens* single-species biofilms prepared as described above. Under scenario 2 (colonization of preexisting *L. monocytogenes* biofilms by *P. fluorescens*, referred to as Lm-Pf biofilms), 20 mL of the *P. fluorescens* inoculum was added into a Petri dish containing SS coupons with *L. monocytogenes* single-species biofilms. In addition, scenario 3 (colonization of SS coupons by the co-culture of *L. monocytogenes* and *P. fluorescens*, referred to as co-culture biofilms) was implemented by inoculating 200 µL of *L. monocytogenes* and *P. fluorescens* working cultures with equal cell numbers (10^8 CFU/mL) into 20 mL of TSB containing sterile SS coupons. All the dual-species biofilms described in the three scenarios were incubated at 15 °C for another 48 h.

2.3. Survival of single- or dual-species biofilm cells during desiccation

After biofilm formation at 15 °C for 48 h, the coupons were washed thrice with PBS and transferred into a new Petri dish, which was placed in a desiccator containing saturated potassium carbonate (Sigma-Aldrich, China) to obtain 43% relative humidity (RH). A data logger (TR300, Amprobe, Germany) was placed in the desiccator to continuously monitor the temperature and RH during storage at 15 °C for 21 days.

At specific time points (days 0, 1, 3, 5, 7, 14, and 21), two coupons from each scenario were sampled to determine the number of surviving cells during desiccation. Each coupon was transferred to a sterile centrifuge tube containing 5 mL of 0.1% peptone water. To detach the biofilm cells from the coupons, the tube was sonicated in an ultrasonic bath (Elma Hans Schimidbauer GmbH &Co., Singen, Germany) at 48 kHz for 3 min, followed by vigorous vortexing for 30 s. This method has been previously shown to effectively detach biofilm cells from SS surfaces (Yang et al., 2015). After 10-fold serial dilutions, 0.1 mL of the cell suspension was spread-plated on trypticase soy agar (TSA; Oxoid) for single-species biofilms, whereas *L. monocytogenes* and *P. fluorescens* in dual-species biofilms were enumerated on *Listeria*-selective agar (PALCAM; Oxoid) or *Pseudomonas* Agar Base supplemented with cetrimide-fucidin-cephalosporin (Oxoid), respectively. The selective agar surfaces were overlaid with TSA (thin-layer agar method) to recover injured cells (Wu, 2008). The *L. monocytogenes* and *P. fluorescens* plates for cell counting were incubated at 37 and 30 °C, respectively, for 48 h.

2.4. Survival of single- or dual-species biofilm cells during disinfection

After biofilm formation at 15 °C for 48 h, the coupons were gently washed thrice with PBS and exposed to 5 mL of 100 µg/mL benzalkonium chloride (BC; Sigma-Aldrich, China) for 15 min. At specific time points (2, 5, 9, 12, and 15 min), two coupons were immediately placed in 5 mL of D/E neutralizing broth (Acumedia, Lansing, MI, USA) to inactivate the disinfectant residues. Tubes with 5 mL of D/E neutralizing broth were then directly subjected to sonication and vortexing as mentioned in subsection 2.3. To enumerate the surviving cells after disinfection, the cell suspension was diluted and spread-plated for cell counting as described above.

2.5. The transfer of L. monocytogenes biofilm cells from SS coupons to salmon fillets

Biofilms were washed thrice with PBS and air-dried for 40 min at room temperature. The method of double meat surface sandwich (the SS coupon was the middle layer between the upper and lower salmon surfaces) (Wang et al., 2015a), with slight modifications, was employed for the transfer of *L. monocytogenes* to salmon fillets. Fresh salmon fillets purchased at a local supermarket served as the recipient surface and were cut into pieces (2 × 1 × 0.5 cm) in a sterile environment. The presence of indigenous *Listeria* spp. was detected by homogenizing a salmon sample (10 g) with 90 mL of sterile buffered peptone water for 2 min and then by plating the homogenate on PALCAM agar. The test-negative samples were used in the following experiment. An SS coupon was placed on a prepared salmon fillet surface and completely covered with another surface of a salmon fillet. Next, a sterilized SS plate weighing 20 g was placed on the upper meat surface for 30 s and removed, in order to apply a consistent pressure on the surface. After the first transfer, the same SS coupon was placed between the second double salmon surfaces, and the procedure was repeated successively for 10 contacts as described above. For each contact, two (upper and lower) salmon fillets were placed in a stomacher bag with 5 mL of 0.1% peptone water, followed by stomaching for 1 min and plating of appropriate serial dilutions on PALCAM agar. The transfer rate (RT) was calculated by means of the following equation (Rodriguez and McLandsborough, 2007):

RT (%) = [Number of transferred cells (CFU/cm^2)/number of cells (CFU/cm^2) in biofilms on SS coupons before transfer] × 100% (1)

2.6. Confocal laser scanning microscopy (CLSM)

Single- and dual-species biofilms (15 °C, 48 h) generated on SS coupons were rinsed with deionized water and simultaneously stained with SYTO9 (Molecular Probes, Eugene, OR, USA) and calcofluor white (250 μg/mL, Sigma). Images were acquired under an inverted FLUOVIEW® FV 1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). The total surface area was scanned with a 10× objective to select three representative areas. Then, the scanned images were obtained at 500 Hz using a 63× water immersion objective with a 488 nm argon laser set to 12% intensity. The emission filters were BA 505–525 for the green channel and BA 430–470 for the blue channel. Representative CLSM images from each coupon were acquired by scanning z-stacks at a scanning step size of 1 μm and were processed in IMARIS 7.6 software (Bitplane AG, Zurich, Switzerland).

2.7. Modeling and statistical analysis

The survival curves of *L. monocytogenes* during desiccation or disinfection were fitted to the Weibull model using the GInaFiT (version 1.6) tool developed by Geeraerd et al. (2005), which is available online (http://cit.kuleuven.be/biotec/downloads.php). The Weibull model is parameterized as follows (Mafart et al., 2002):

$$\log(N) = \log(N_0) - [(t/\text{delta})^{p}] \qquad (2)$$

where delta is the time to the first decimal (log) reduction, N_0 is the initial biofilm cell density, t denotes time, and *p* is the shape parameter.

The transfer curves of *L. monocytogenes* were fitted to a typical logistic model, which was demonstrated to fit the process of transfer of biofilm cells accurately (Wang et al., 2015a). The equation is parameterized as

$$Y = P/(1 + C \times e^{-r.X}) \qquad (3)$$

where X is the contact succession number, and Y is the number (log CFU/cm^2) of cells transferred from a biofilm to salmon at succession number X. The biological meaning of the parameters was redefined as follows: *P* is the value of an asymptote, reflecting cell persistence on the surface, and r is the specific detachment coefficient (t^{-1}) related to the strengths of cell attachment (Herrera et al., 2007). The fitting performance of the model was statistically evaluated based on R^2 and MSE_{model}.

All mean values were calculated from the results of three independent experiments with duplicate samples (n = 6). Statistical analysis was conducted by one-way analysis of variance (ANOVA), and the mean values were compared by Duncan's multiple-range test in the SPSS software (Statistical Package for the Social Sciences, version 17.0, IBM, NY, USA). The estimates of Weibull model parameters were compared by Tukey's honest significant difference (HSD) test at 5% significance.

Table 1
Initial biofilm populations (log CFU/cm^2)† of single-species biofilms of *L. monocytogenes* (Lm) and *P. fluorescens* (Pf) and dual-species biofilms formed at 15 °C for 48 h via colonization by co-culture of *L. monocytogenes* and *P. fluorescens* (co-culture), colonization of preexisting *P. fluorescens* biofilms by *L. monocytogenes* (Pf-Lm), or colonization of preexisting *L. monocytogenes* biofilms by *P. fluorescens* (Lm-Pf).

Type of biofilm	Bacteria	Initial biofilm population (Log CFU/cm^2)	Proportion in the dual-species biofilm
Single	*L. monocytogenes*	7.21 ± 0.21^b	–
	P. fluorescens	7.58 ± 0.16^a	–
Co-culture	*L. monocytogenes*	6.92 ± 0.22^c	10.69
	P. fluorescens	7.82 ± 0.22^a	89.21
Lm-Pf	*L. monocytogenes*	6.78 ± 0.21^c	8.94
	P. fluorescens	7.79 ± 0.23^a	91.06
Pf-Lm	*L. monocytogenes*	6.96 ± 0.08 ^{c}	13.06
	P. fluorescens	7.77 ± 0.17 ^{a}	86.93

†Values are expressed as mean log CFU/cm^2 ± standard deviation (n = 6). Values with different lowercase (a, b) letters in the same column are significantly ($P < 0.05$) different.

3. Results

3.1. Initial populations in single- and dual-species biofilms

The populations of *L. monocytogenes* and *P. fluorescens* in single- and dual-species biofilms formed at 15 °C for 48 h are described in Table 1. For single-species biofilms, the cell counts of *L. monocytogenes* and *P. fluorescens* reached 7.21 ± 0.21 and 7.58 ± 0.16 log CFU/cm^2, respectively. In dual-species biofilms, *P. fluorescens* attained approximately one log CFU/cm^2 higher cell density compared to *L. monocytogenes*, thus constituting approximately 90% of the total population. Biofilm cell densities of *L. monocytogenes* in dual-species biofilms reached 6.78 ± 0.21 to 6.95 ± 0.45 log CFU/cm^2 and were significantly lower ($P < 0.05$) than those of single-species biofilms. However, there was no significant difference ($P > 0.05$) in *P. fluorescens* biofilm cell counts between single- and dual-species biofilms. In addition, there was no significant difference ($P > 0.05$) in biofilm population level of *L. monocytogenes* or *P. fluorescens* among dual-species biofilms resulting from different colonization sequences.

3.2. Resistance of *L. monocytogenes* and *P. fluorescens* to desiccation in single- and dual-species biofilms

After the onset of 3-week desiccation, the populations of *L. monocytogenes* and *P. fluorescens* in single-species biofilms decreased from 7.21 ± 0.21 to 3.36 ± 0.29 and from 7.58 ± 0.16 to 3.98 ± 0.49 log CFU/cm^2, respectively, indicating similar sensitivity to desiccation (Fig. 1). The viability loss of *L. monocytogenes* in single-species biofilms (3.85 ± 0.25 log CFU/cm^2) was significantly higher ($P < 0.05$) as compared to dual-species biofilms, which yielded the log reduction of 2.38 ± 0.25, 1.77 ± 0.40, and 2.50 ± 0.25 log CFU/cm^2 in co-culture, Pf-Lm, and Lm-Pf dual-species biofilms, respectively (Fig. 1A). However, a similar trend of *P. fluorescens* survival curves was observed, with a surviving population of 3.28–4.34 log CFU/cm^2 in dual-species biofilms and 3.98 ± 0.49 log CFU/cm^2 in single-species biofilms (Fig. 1B).

The non-linear survival curves were adequately fitted using the

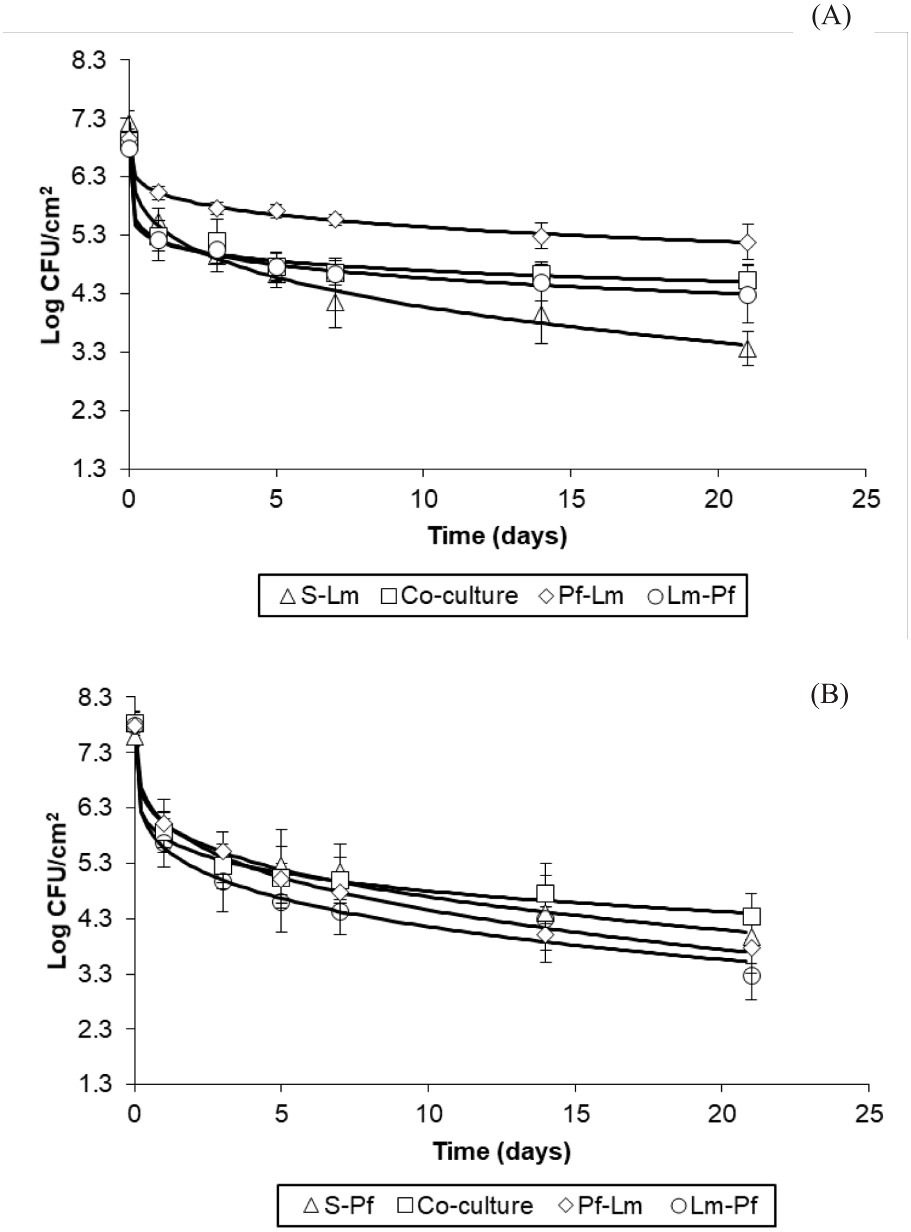

Fig. 1. Survival of single- and dual-species biofilms (15 °C, 48 h) on SS coupons during exposure to 43% RH at 15 °C for 21 days. *L. monocytogenes* (A) and *P. fluorescens* (B) single-species (S-) and dual-species biofilms formed via three types of colonization sequences (co-culture, Pf-Lm, or Lm-Pf). Symbols are averages of survivor counts (n = 6), and error bars indicate standard deviations. The lines in the graph represent the number of surviving cells predicted by the Weibull model fits. The detection limit of the experiment was 1.3 log CFU/cm^2.

Weibull model as indicated by the statistical analysis of model fits (Table 2). As shown by the delta value (time to first log reduction), *L. monocytogenes* was sensitive to desiccation, with 90% of the population being inactivated in less than 0.5 day in both single-species and dual-species biofilms, except for Pf-Lm dual-species biofilms, which had a significantly higher ($P < 0.05$) delta value: 1.45 ± 0.57. *P. fluorescens* yielded a delta value of 0.21 ± 0.16, which was not significantly different ($P > 0.05$) from the values in dual-species biofilms, indicating that the sensitivity of *P. fluorescens* to desiccation was not affected by the presence of *L. monocytogenes*.

3.3. Resistance of *L. monocytogenes* and *P. fluorescens* to disinfection in single- and dual-species biofilms

In single-species biofilms, BC treatment for 15 min decreased the number of *L. monocytogenes* and *P. fluorescens* cells to 1.79 ± 0.27 and 5.19 ± 0.37 log CFU/cm^2, respectively, indicating that *L. monocytogenes* was more sensitive to the disinfectant than *P. fluorescens* (Fig. 2). *L. monocytogenes* was less sensitive to BC in dual-species biofilms compared to single-species biofilms, with the surviving cell numbers being 5.02 ± 0.26, 5.73 ± 0.26, 4.73 ± 0.56, and 1.79 ± 0.27 log CFU/cm^2 in co-culture, Pf-Lm, Lm-Pf, and single-species biofilms, respectively (Fig. 2A). Similarly, the survivor population of *P. fluorescens* in dual-species biofilms ranging from 6.37 ± 0.30 to 6.76 ± 0.38 log CFU/cm^2 was significantly larger ($P < 0.05$) than that of the survivor cells (5.19 ± 0.37 log CFU/cm^2) in single-species biofilms (Fig. 2B).

Because there was no significant reduction (unpaired-*t* test, $P > 0.05$) in the populations of *P. fluorescens* in dual-species biofilms after exposure to BC for 15 min, only the survival curves of *L.*

Table 2

Inactivation kinetics[†] observed during desiccation (43% RH, 15 °C, 21 d) of single-species biofilms of *L. monocytogenes* (Lm) and *P. fluorescens* (Pf), and dual-species biofilms formed via colonization by co-culture of *L. monocytogenes* and *P. fluorescens* (co-culture), colonization of preexisting *P. fluorescens* biofilms by *L. monocytogenes* (Pf-Lm), or colonization of preexisting *L. monocytogenes* biofilms by *P. fluorescens* (Lm-Pf).

Biofilm type	Bacteria	Model parameter estimates		Statistical indices of model fit	
		Delta (days)	*P* (days)	R^2	MSE model
Single	*L. monocytogenes*	0.11 ± 0.07^b	0.25 ± 0.03^b	0.90	0.16
	P. fluorescens	0.21 ± 0.16^b	0.27 ± 0.04^b	0.85	0.23
Co-culture	*L. monocytogenes*	0.01 ± 0.01^b	0.11 ± 0.02^c	0.90	0.07
	P. fluorescens	0.01 ± 0.02^b	0.17 ± 0.03^c	0.86	0.20
Pf-Lm	*L. monocytogenes*	1.45 ± 0.57^a	0.22 ± 0.03^b	0.91	0.03
	P. fluorescens	0.14 ± 0.08^b	0.28 ± 0.03^b	0.92	0.39
Lm-Pf	*L. monocytogenes*	0.06 ± 0.07^b	0.16 ± 0.03^c	0.88	0.30
	P. fluorescens	0.03 ± 0.03^b	0.33 ± 0.03^a	0.88	0.50

[†] Values in the same column followed by different letters are significantly ($P < 0.05$) different from each other.

monocytogenes biofilms were adequately fitted to the Weibull model (Table 3). The delta value of *L. monocytogenes* in single-species biofilms was 0.02 ± 0.01, whereas it was 0.92 ± 0.59, 11.30 ± 1.82, and 7.08 ± 1.98 in Lm-Pf, Pf-Lm, and co-culture dual-species biofilms, respectively. These results indicated that the resistance of *L. monocytogenes* biofilm cells was greater in the presence of *P. fluorescens*, with the highest resistance observed in Pf-Lm biofilms among dual-species biofilms.

3.4. *Transfer of* L. monocytogenes *from single- and dual-species biofilms on SS surfaces to salmon fillets*

To determine the transfer efficiency of *L. monocytogenes* cells from single- and dual-species biofilms on SS coupons to salmon fillets, the transfer rate was calculated based on single-contact transfer. A significantly higher ($P < 0.05$) transfer rate (58.0%) was observed for *L. monocytogenes* in single-species biofilms than in co-culture, Pf-Lm, and Lm-Pf dual-species biofilms (5.3%, 3.6%, and 10.3%, respectively; Fig. 3).

The transfer curves of *L. monocytogenes* biofilms to 10 slices of salmon fillets are presented in Fig. 4. A progressive reduction trend was observed when the frequency of contact increased, with the transferred cell number decreasing from 6.60 ± 0.44 to 4.62 ± 0.49 log CFU/cm^2 in single-species biofilms, from 5.78 ± 0.32 to 4.13 ± 0.37 log CFU/cm^2 in co-culture biofilms, from 5.49 ± 0.29 to 4.22 ± 0.33 log CFU/cm^2 in Pf-Lm biofilms, and from 5.72 ± 0.29 to 3.81 ± 0.29 log CFU/cm^2 in Lm-Pf biofilms. The parameters obtained by fitting the transfer curves to the logistic model are given in Table 4. A significantly higher ($P < 0.05$) *P* value was observed for single-species biofilms than for dual-species biofilms with different colonization sequences, indicating that higher counts of *L. monocytogenes* in single-species biofilms remained on the coupon after 10 successive contacts with salmon. In addition, Pf-Lm biofilms yielded a significantly lower ($P < 0.05$) r value as compared with single-species and Lm-Pf biofilms, indicating stronger adhesion of *L. monocytogenes* cells to the contact surface when they get attached to preexisting *P. fluorescens* biofilms.

3.5. *Structure of* L. monocytogenes *and* P. fluorescens *single- and dual-species biofilms*

CLSM was performed to investigate the spatial organization of the biofilms and the distribution of EPS. Calcofluor white was used to stain cellulose and other polysaccharides present in the EPS, whereas SYTO9 was utilized to stain all the cells in the biofilms. *L. monocytogenes* in single-species biofilms formed a thin-layer structure with weak calcofluor fluorescence (Fig. 5A). In contrast, scattered microcolonies covered with polysaccharides were observed in *P. fluorescens* single-species biofilms, which were much thicker than those of *L. monocytogenes* (Fig. 5B). Co-culture biofilms had a flat multilayered structure with a maximum thickness of 5 μm, and only a few small clusters were surrounded by polysaccharides (Fig. 5C). Pf-Lm biofilms formed a complex three-dimensional structure with a maximum thickness of approximately 10 μm. Greater amounts of polysaccharides were distributed over the z-stack in Pf-Lm biofilms than in co-culture and Lm-Pf dual-species biofilms (Fig. 5D). When *L. monocytogenes* was the first colonizer, Lm-Pf biofilms developed a thin-layer structure, which was denser than that of *L. monocytogenes* single-species biofilms, and only small amounts of polysaccharides were observed (Fig. 5E).

4. Discussion

This study evaluated the effect of colonization sequences of *L. monocytogenes* and *P. fluorescens* dual-species biofilms on *L. monocytogenes* survival after exposure to food-related stresses (desiccation and disinfection) as well as *L. monocytogenes* behavior during transfer to salmon products. To simulate a salmon processing environment, food grade SS surfaces and low temperature (15 °C) were selected as the conditions for biofilm formation. Forty-eight hours of incubation was chosen for biofilm formation because the biofilms grown for 48 h reached cell density above 7.0 log CFU/cm^2 and developed typical biofilm structure as determined by CLSM. Three colonization sequences (co-culture, Pf-Lm, and Lm-Pf) represent three scenarios of *L. monocytogenes* and *P. fluorescens* co-existence in food processing environments. The co-culture condition mimics biofilm formation that starts from the simultaneous attachment of *L. monocytogenes* and *P. fluorescens* planktonic cells (derived from the same source such as salmon) to inert surfaces (Møretrø et al., 2016). Pf-Lm and Lm-Pf represent dual-species biofilm formation resulting from *L. monocytogenes* or *P. fluorescens* attachment respectively to a preexisting *P. fluorescens* or *L. monocytogenes* biofilm, which remain in specific niches without sufficient cleaning and disinfection.

After 48 h incubation, *L. monocytogenes* and *P. fluorescens* produced mature single-species biofilms with the population density above 7.0 log CFU/cm^2 and formed different three-dimensional structures according to CLSM (Fig. 5A and B). Irrespective of the colonization sequence, cell density of *L. monocytogenes* in dual-species biofilms was lower than that in single-species biofilms. This finding is consistent with the results of other studies, which indicate lower adherence levels of *L. monocytogenes* in the co-culture with *Pseudomonas putida* (Giaouris et al., 2013; Ibusquiza et al., 2012). However, some studies reported stronger attachment of *L. monocytogenes* to the surfaces with preexisting *Pseudomonas* biofilms than to *Pseudomonas*-free surfaces (Hassan et al., 2004; Puga et al., 2018). These contradictory results could be due to individual *Pseudomonas* strain properties, such as the EPS-producing ability. Carpentier and Chassaing (2004) found that some *Pseudomonas* strains could decrease the biofilm density of *L. monocytogenes*, whereas other *Pseudomonas* strains had no effect on its attachment level. In addition, the present results suggest that cell density of *P. fluorescens* was not affected by the presence of *L. monocytogenes* and that *P. fluorescens* constituted 90% of the dual-species biofilm population, in agreement with the results of Chorianopoulos et al. (2008), who reported that *Pseudomonas* was the dominant species in mixed-species biofilms. Overall, these results indicate the competitive advantage of *Pseudomonas* over *L. monocytogenes* in dual-species biofilm formation.

The present results showed that the resistance of *L. monocytogenes* biofilm cells to desiccation was enhanced by the presence of *P. fluorescens*, irrespective of the colonization sequence. Similarly, Bremer et al. (2001) observed enhanced desiccation survival of *L. monocytogenes* biofilm cells in the presence of *Flavobacterium* spp. It is well

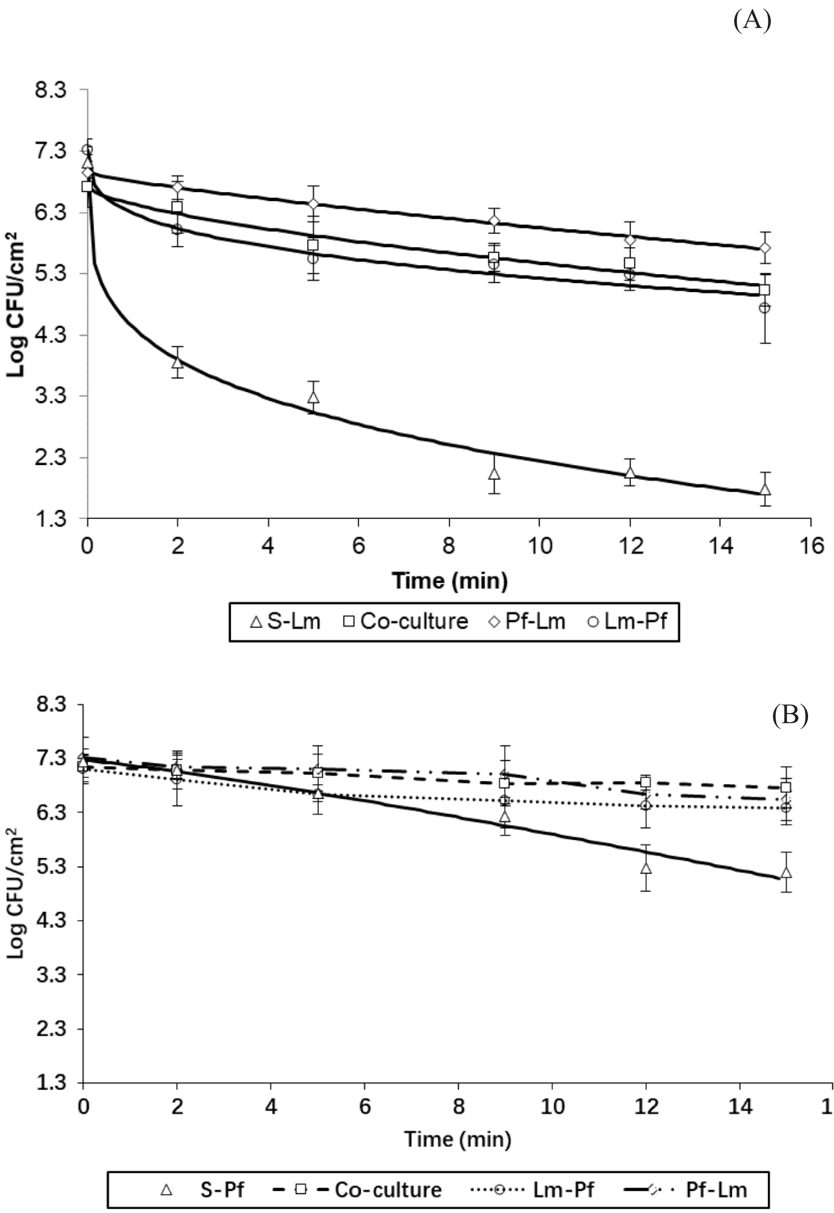

Fig. 2. Survival of single- and dual-species biofilms (15 °C, 48 h) on SS coupons exposed to benzalkonium chloride (BC, 100 μg/mL) for 15 min. *L. monocytogenes* (A) and *P. fluorescens* (B) single-species (S-) and dual-species biofilms formed via three types of colonization sequences (co-culture, Pf-Lm, or Lm-Pf). Symbols are averages of survivor counts (n = 6), and error bars indicate standard deviations. The lines in graph (A) and *P. fluorescens* in single-species biofilms in graph (B) represent the number of surviving cells predicted by the Weibull model fits. The detection limit of the experiment was 1.3 log CFU/cm^2.

Table 3
Inactivation kinetics[†] of *L. monocytogenes* (Lm) during disinfection (benzalkonium chloride, BC; 15 min) in single species and dual-species biofilms formed via colonization by co-culture of *L. monocytogenes* and *P. fluorescens* (co-culture), colonization of preexisting *P. fluorescens* biofilms by *L. monocytogenes* (Pf-Lm), or via colonization of preexisting *L. monocytogenes* biofilms by *P. fluorescens* (Lm-Pf).

Biofilm type	Model parameter estimates		Statistical indices of model fit	
	Delta (min)	*P* (min)	R^2	MSE model
Single	0.02 ± 0.01^{d}	0.26 ± 0.02^{b}	0.97	0.11
Co-culture	7.08 ± 1.98^{b}	0.64 ± 0.17^{a}	0.72	0.14
Pf-Lm	11.30 ± 1.82^{a}	0.80 ± 0.20^{a}	0.74	0.08
Lm-Pf	0.92 ± 0.59^{c}	0.31 ± 0.07^{b}	0.83	0.37

[†] Values in the same column followed by different letters are significantly ($P < 0.05$) different from each other.

known that *Pseudomonas* spp. is a strong biofilm former, the EPS of which mainly consists of polysaccharides and proteins (Mann and Wozniak, 2012). Thus, it was speculated that the EPS produced by *P. fluorescens* could protect *L. monocytogenes* against a dry environment. However, it was previously reported that *P. fluorescens* increased the inactivation of *L. monocytogenes* during desiccation, although more EPS in dual-species biofilms could explain the increase in the first decimal reduction time of listerial subpopulation 1 (Daneshvar Alavi and Truelstrup Hansen, 2013). In their study, TSB-glucose led to acid tolerance response of *L. monocytogenes* in single-species biofilms, and this arrangement enhanced its desiccation survival. On the other hand, this was not the case in dual-species biofilms because *P. fluorescens* was the dominant species (99%), and thus the culture medium could not be acidified. In addition, the present study showed that co-culture with *L. monocytogenes* did not affect desiccation survival of *P. fluorescens*. This finding may be explained by the dominance of *P. fluorescens* in dual-species biofilms, and therefore it contributes most of the biofilm matrix.

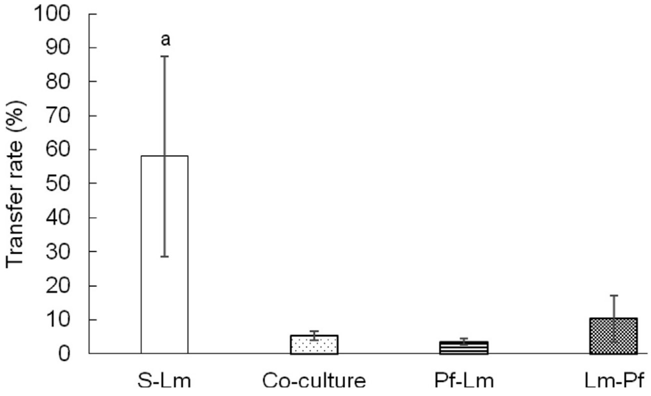

Fig. 3. Transfer rates of *L. monocytogenes* in single-species (S-Lm) and dual-species biofilms inoculated with *P. fluorescens* via three types of colonization sequences (co-culture, Pf-Lm, or Lm-Pf) from SS coupons to salmon fillets. All measurements were performed in triplicate, and all bars show mean ± standard deviation. Bars followed by different letters are significantly different ($P < 0.05$), $n = 6$.

Resistance to BC is a cause for food safety concern because BC is a member of quaternary ammonium compounds that are extensively used as disinfectants at food processing plants. Results revealed that survival of *L. monocytogenes* and *P. fluorescens* in dual-species biofilms was enhanced compared to respective single-species biofilms during exposure to BC. An increasing body of evidence suggests that mixed-species biofilms have greater resistance to disinfectants. Previous studies reported that co-culture with *Pseudomonas* spp. can increase disinfection resistance of *L. monocytogenes* (Ibusquiza et al., 2012) and *Salmonella* spp. (Pang et al., 2017), which is possibly related to the complicated three-dimensional biofilm structure and higher EPS production.

During exposure to desiccation and disinfectant, the higher surviving cell number and lower inactivation rate of *L. monocytogenes* were observed when it was incorporated into the preexisting *P. fluorescens* biofilms (Pf-Lm) in comparison with co-culture and Lm-Pf dual-species biofilms. These results indicate that the colonization sequence of *P. fluorescens* and *L. monocytogenes* during biofilm formation affects the response of *L. monocytogenes* to environmental stresses. Furthermore, these results raise serious concerns about the pre-formed *P. fluorescens* biofilms that remain on the surfaces after daily cleaning and disinfection. Enhanced EPS production by previously formed *P. fluorescens* biofilms for 48 h could be one of the reasons underlying the enhanced survival of *L. monocytogenes* in Pf-Lm biofilms. This notion is supported by the CLSM observations, which showed larger amounts of polysaccharides in Pf-Lm biofilms than in Lm-Pf and co-culture dual-species biofilms (Fig. 5). EPS plays an important role in the desiccation survival of biofilm cells because it creates a water-rich gel around the bacterial cells (Esbelin et al., 2018). Furthermore, EPS acts as a physical shield that limits disinfectant penetration, thereby hampering its efficacy (Bridier et al., 2011). Consequently, these results indicate that pre-existing *P. fluorescens* biofilms might provide more EPS to *L. monocytogenes* as the second colonizer and protect it from desiccation and disinfection.

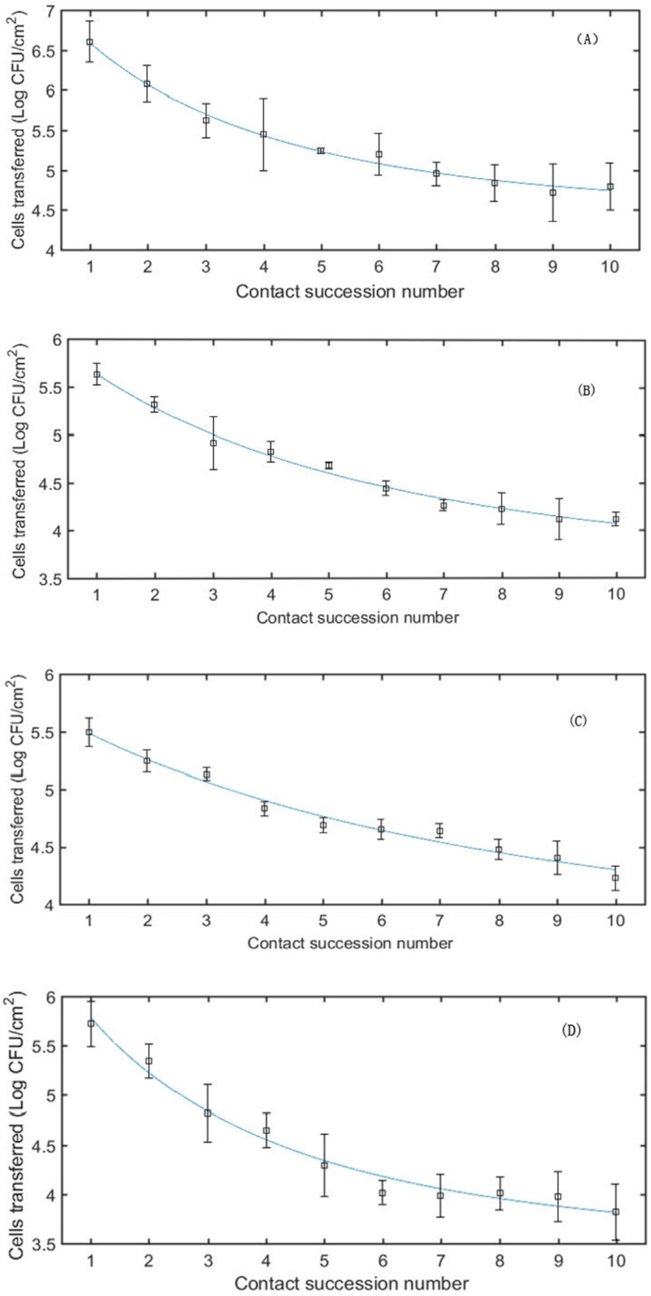

Fig. 4. The transfer (log CFU/cm^2) of *L. monocytogenes* biofilm cells from SS coupons containing single-species (A) or dual-species biofilms inoculated via different colonization sequences [co-culture condition (B), Pf-Lm (C), or Lm-Pf (D)] to 10 slices of salmon fillets. Symbols are averages of transferred cell counts ($n = 6$), and error bars denote standard deviations. The lines in the graph represent the transferred cell number predicted by typical logistic model fits.

In cold-smoked salmon processing plants, *L. monocytogenes* contamination mainly occurs during processing, with slicing machines as a reservoir for *L. monocytogenes* (Vogel et al., 2001). Biofilm cells formed on the slicer surface could lead to cross-contamination by transmission during slicing. Therefore, investigating the transfer of *L. monocytogenes* biofilms to food products is of utmost importance. It was found here that 58% of *L. monocytogenes* populations in single-species biofilms was transferred to salmon fillets by a single contact within 30 s. Similar transfer efficiency (50–60%) of *L. monocytogenes* biofilm cells from SS coupons to food products has been reported elsewhere (Hansen and Vogel, 2011; Rodriguez and McLandsborough, 2007). Additionally, *L. monocytogenes* had a lower transfer rate in dual-species biofilms, regardless of the colonization sequence. Similarly, Al-Adawi et al. (2016) reported reduced transfer efficiency of *Salmonella* Typhimurium and *Pseudomonas aeruginosa* in mixed-species biofilms with *E. coli* to chicken as compared to single-species biofilms. The transfer process can be considered to consist of successive biofilm detachment steps due to direct surface-to-surface contact. Transfer kinetics showed that higher counts of *L. monocytogenes* in single-species biofilms remained on the coupon after 10 successive contacts in comparison with dual-species biofilms, possibly owing to high initial cell density in single-species biofilms. The lower detachment rate of *L. monocytogenes* in Pf-Lm

Table 4
Parameters† of transfer models for biofilms formed by *L. monocytogenes* in single-species (S-Lm) and dual-species biofilms via colonization by co-culture of *L. monocytogenes* and *P. fluorescens* (co-culture), colonization of preexisting *P. fluorescens* biofilms by *L. monocytogenes* (Pf-Lm), or via colonization of preexisting *L. monocytogenes* biofilms by *P. fluorescens* (Lm-Pf).

Biofilm type	Model parameter estimates			Statistical indices of model fit	
	P	C	r	R^2	MSE model
S-Lm	4.41 ± 0.45^a	−0.40 ± 0.06^a	0.21 ± 0.08^a	0.82	0.02
Co-culture	3.58 ± 0.19^b	−0.46 ± 0.04^a	0.12 ± 0.02^{ab}	0.82	0.01
Pf-Lm	3.54 ± 0.04^b	−0.27 ± 0.01^b	0.09 ± 0.04^b	0.92	0.02
Lm-Pf	3.65 ± 0.09^b	−0.44 ± 0.02^a	0.20 ± 0.01^a	0.87	0.02

†Values in the same column followed by different letters are significantly ($P < 0.05$) different from each othe.

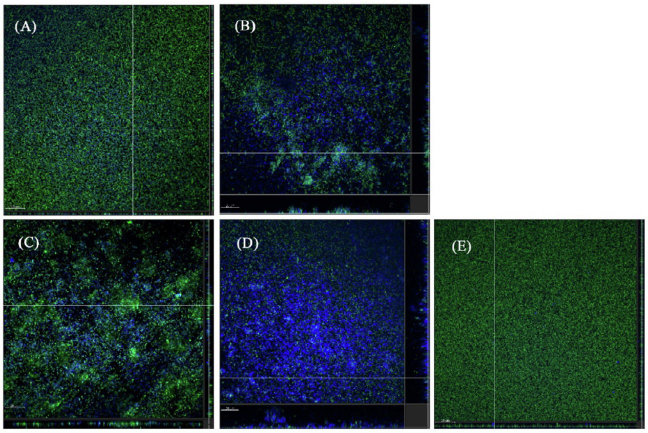

Fig. 5. CLSM images of section views of 48 h single-species biofilms of *L. monocytogenes* (A) or *P. fluorescens* (B) and 48 h dual-species biofilms formed via different colonization sequences: the co-culture condition (C), Pf-Lm (D), or Lm-Pf (E). Cells are stained with SYTO9 (green), and exopolysaccharides are stained with calcofluor (blue). The top and side images of each panel represent the x-z and y-z planes respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biofilms can be explained by greater polysaccharides production as compared with single-species biofilms and Lm-Pf biofilms (Fig. 5). One recent study also reported matrix overproduction by pre-formed *P. fluorescens* biofilms after colonization by *L. monocytogenes* (Puga et al., 2018). EPS is critical for maintaining the stability and integrity of biofilm structure via cellular adhesion and cohesion (Simoes et al., 2005). It has been reported that cohesive energy of biofilms strongly correlates with polysaccharide concentration (Ahimou et al., 2007a). Interestingly, Puga et al. (2018) observed that *L. monocytogenes* could invade previously formed *P. fluorescens* biofilms and preferentially occupy the bottom layers in dual-species biofilms. Considering the possible spatial organization, *L. monocytogenes* may be located in the deep layer of Pf-Lm biofilms, which had much thicker structure than that of Lm-Pf biofilms in the present study. Biofilm cohesion increases with the biofilm depth (Ahimou et al., 2007b; Derlon et al., 2008), resulting in the strong attachment of *L. monocytogenes* via pre-formed *P. fluorescens* biofilms.

5. Conclusion

The effect of colonization sequences of *L. monocytogenes* and *P. fluorescens* during biofilm formation on the resistance of *L. monocytogenes* to desiccation and disinfectant was investigated, as well as *L. monocytogenes* transfer to salmon products. Composition of the dual-species biofilm population was not affected by the colonization sequence. The pre-formed *P. fluorescens* biofilms (Pf-Lm) enhanced survival of *L. monocytogenes* biofilm cells subjected to desiccation and disinfection, but the transfer rate of *L. monocytogenes* in Pf-Lm biofilms to salmon was reduced by strengthening their attachment to the surfaces, relative to dual-species biofilms with other colonization sequences. This finding is possibly related to the thick three-dimensional structure with a large amount of EPS in Pf-Lm biofilms. Thus, this study highlights the protective effect of pre-formed *P. fluorescens* biofilms on the persistence of *L. monocytogenes* on food contact surfaces, suggesting that an improved strategy to control and eradicate *L. monocytogenes* biofilms is needed.

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