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Interactions between spoilage bacteria in tri-species biofilms developed under simulated meat processing conditions.

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ABSTRACT

The formation of biofilms in the food industry is a major issue, as they are a frequent source of contamination of products, which can result in significant economic losses for processors through spoilage of foods or pose serious health concerns for consumers when foodborne pathogens are present. In this study, experiments were carried out using CDC Biofilm Reactors to produce biofilms on two test surfaces (polystyrene and stainless steel coupons) under a regimen for simulated meat processing conditions (SMPC). This entailed a 12 day regimen of daily cycles of filling the reactors with a meat slurry and letting stand for 16 h, followed by draining and refilling with water for an 8 h period in order to mimic a possible scenario of fluctuating periods of nutrient availability and starvation in a meat processing facility. Strains of *Pseudomonas fluorescens*, *Lactobacillus plantarum* and *Leuconostoc pseudomesenteroides* were used for mono and mixed cultures biofilms as they are relevant spoilage bacteria in the meat processing industry. In monoculture, the viable cell densities (CFU/cm^2) of the two lactic acid bacteria species tested were higher for biofilms grown on polystyrene as compared to those obtained on stainless steel, whereas viable cell numbers in *P. fluorescens* monoculture were surface-independent. Synergistic interactions were demonstrated during growth of multi-species biofilms. Results from experiments where one of the 3 strains was inoculated 24 h before introduction of the other two strains showed increased levels of *L. plantarum* within biofilms grown on both test surfaces. The model developed here serves as a baseline to study the interactions between potential spoilage bacteria during biofilm development.

1. Introduction

In food processing industries, the ability of microorganisms to attach to solid surfaces is an important issue (Mafu et al., 2002; Srey et al., 2013). Biofilms are complex communities of microbial cells adhering to, and growing on a substratum, and are often encased in a matrix of extracellular polymeric substances (Parkar et al., 2004). This matrix provides added protection to the resident microflora against harsh conditions, including exposure to cleaners (Araújo et al., 2011; Corcoran et al., 2014) and antimicrobial agents (Donlan, 2002). Therefore, the capacity to form biofilm is a key factor that could explain the persistence of pathogenic and spoilage bacteria in food factory environments (Annous et al., 2009; Vestby et al., 2009). Bacterial cells can be disseminated from biofilms (Parkar et al., 2004) and may become a perpetual source of contamination for food products or food contact surfaces even though decontamination processes and strict hygiene controls are in place. This may result in significant economic losses due to food spoilage (Kumar and Anand, 1998; Brooks and Flint, 2008) and increase the risk of outbreaks of foodborne illnesses (Coughlan et al., 2016).

Most studies on biofilm have focused on a single species of bacteria (Burmølle et al., 2006), often at an early stage of adhesion (usually few h to 24–48 h) and in aqueous suspensions or in microbiologic culture broths (Carpentier and Chassaing, 2004). However, in the real world bacteria will generally grow on surfaces in competition with other microorganisms (Abee et al., 2011). Most mature biofilms are inhabited by numerous microbial species living in close proximity to one another and their interactions may have positive or negative effects on other resident microorganisms. Synergistic interactions in multi-species biofilms can enhance biofilm formation (Burmølle et al., 2006; Castonguay et al., 2006) and increase the resistance of cells to inimical environmental conditions (Burmølle et al., 2006; Simões et al., 2009; Lee et al., 2014). Moreover, the presence of certain species of microorganisms on a surface can promote the adhesion of other species (Cloete et al., 2009; Klayman et al., 2009b). Competitive interactions can be antagonistic by the limitation of nutrient sources, oxygen, available space to colonize

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(Giaouris et al., 2015) and/or through the production of compounds, such as bacteriocins and organic acids, that may inactivate or inhibit the growth of other species. These negative interactions between different species represent different forms of "amensalism" (Rendueles and Ghigo, 2012). There is a lack of knowledge regarding the fate of spoilage microorganisms within complex mature multi-species biofilms formed under environmental conditions simulating those encountered in food processing facilities. Mimetic conditions for such models should include alternation of periods of food soiling, starvation and desiccation. The development of model multi-species biofilms formed by representative food spoilage bacteria under simulated processing plant conditions and the evaluation of their persistence would lead to a better understanding of biofilm formation capabilities of food spoilage bacteria. Also, information gathered from studies employing such models could be essential for the design of novel processing equipment and the formulation of effective cleaning/sanitization strategies.

In this context, the aim of the present study was to develop and characterize model mature multi-species biofilms formed under conditions that mimic cycling of nutrient rich and starvation periods encountered between routine cleaning and soiling events in meat processing facilities. CDC Biofilm Reactors from Biosurface Technologies Corporation were used to grow mature biofilms under "simulated meat processing conditions" (SMPC) (Lapointe et al., 2016). The SMPC employed here involved a dynamic 12-day regimen of alternating periods of nutrient availability and starvation, interrupted midway by a two day "shutdown" (weekend), in order to mimic two weeks of processing line activity. To assess the impact of multi-species interactions on biofilm formation three common meat spoilage bacteria (*Pseudomonas fluorescens*, *Lactobacillus plantarum* and *Leuconostoc pseudomesenteroides*) were studied. Single-species biofilms for each test strain were initially examined for comparison of their behaviors when residing in multi-species biofilms. Triple-species biofilms were produced on stainless steel (SS) and polystyrene (PS) surfaces by simultaneously introducing each of the three representative spoilage strains into a reactor (co-cultivation) or by pre-colonization of the target surface with a single species prior to the introduction of the two other bacterial strains.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Pseudomonas fluorescens ATCC 13525, *Lactobacillus plantarum* ATCC 10241 and *Leuconostoc pseudomesenteroides* ATCC 12291 were used in this study. The L. *plantarum* and *L. pseudomesenteroides* strains were initially propagated in Lactobacillus MRS broth (BD, Mississauga, ON, Canada) and *P. fluorescens* in Tryptic Soy broth (TSB - BD). Stock cultures were prepared by growing cells overnight at optimal temperatures, concentrating by centrifugation and re-suspending in Brain Heart Infusion broth (BHI - BD) supplemented with 20% glycerol (*v/v*) (Laboratoire Mat, Qc, Canada). Strains were then frozen at −80 °C in sterile cryogenic vials. Before each independent assay, fresh pure cultures were prepared by adding 0.4 ml thawed stock cultures to 40 ml of MRS or TSB broth and incubating at 30 °C for 20 h. *Pseudomonas* cultures were grown under low agitation. At the end of the incubation period, the pH was determined and the pure cultures were centrifuged at 4000 × *g* for 20 min at 4 °C. Cells were washed, re-suspended in saline (0.85% NaCl) and diluted in order to reach the desired initial bacterial population density in the reactor.

2.2. Meat slurry

Meat slurry (MS) was prepared by homogenizing 400 g of fully-cooked sliced roast beef in a 10-fold volume of deionized water using a PowerGen 700 homogenizer equipped with a 35 mm diameter generator probe (Thermo Fisher Scientific, Mississauga, ON). The MS was filtered through eight layers of cheese cloth (85 weave) and then autoclaved for 30 min at 121 °C. Following cooling to room temperature, the MS was filtered through a Whatman 591 filter to remove coarse heat-generated precipitates and then dispensed into appropriate aliquots and stored frozen until use. Just prior to each independent assay, MS stocks were completely thawed and then supplemented with 30 g/L enzymatic meat digest (HiMedia Laboratories PVT Ltd., Mumbai, India), 15 g/L glucose (Sigma, MO, USA) and 22.06 g/L sodium citrate dihydrate (EMD, Etobicoke, ON, Canada) in order to stimulate growth of lactic acid bacteria. Once dissolved, the fortified MS was diluted 10 fold with deionized water and then autoclaved for 20 min at 121 °C. The resulting diluted modified meat slurry medium was called MS+ and was used for all biofilm reactor experiments throughout this study.

2.3. CDC biofilm reactor preparation

Three CDC biofilm reactors (#CBR 90-2; Biosurface Technologies Corporation, Bozeman, MT, USA), each fitted with 8 rod holders containing either three polystyrene or stainless steel (grade 304) 12.7 mm diameter coupons (#RD128-PS and RD128-304; Biosurface Technologies Corporation) were used for the production of biofilms. The selection of test surface materials was based on the following: stainless steel (SS) is one of the most frequently employed materials in the manufacture of food processing equipment and polystyrene (PS) is one of the most popular polymers used for food packaging (Pimentel Filho et al., 2014). Those materials were also selected based on their surface properties as SS is hydrophilic and PS, hydrophobic.

Biofilms were developed on the coupons while exposed to shear forces created by the rotating paddle at the bottom of the reactors (Williams and Bloebaum, 2010). Before use, biofilm reactors were cleaned, with a 1% Tergazyme solution (Alconox, White Plains, NY, USA) for a minimum of 1 h and under a vigorous agitation. The reactors were then rinsed thoroughly with deionized water, soaked for a minimum of 1 h with a 0.5 N NaOH solution (pH 11), then thoroughly rinsed again with deionized water and sterilized in an autoclave. The coupons were washed and sonicated for 10 min in an ultrasonic water bath (Branson 5510 (Danbury, CT, USA), 40 kHz) in the same cleaning solutions used for the reactors, rinsed thoroughly with deionized water after each cleaning step and allowed to air dry in a biological safety cabinet prior to use. The SS coupons were then autoclaved directly on the rods in the reactors. The PS coupons were sterilized by irradiation (25 kGy) at room temperature under inert conditions (N_2) using a Nordion 651 PT Gamma beam irradiator. The PS coupons were further inserted in the rods under sterile conditions.

2.4. Simulated meat processing conditions (SMPC)

All biofilm reactor trials were synchronized to begin on the first "working day" of the week (Monday). For the initial monoculture trials each reactor was first filled with a sterile 0.85% NaCl solution and then inoculated with one of the fresh (20 h) pure cultures to obtain initial bacterial population densities of 1×10^7 CFU/ml. For the multi-species biofilm experiments all three of selected strains were standardized and then simultaneously introduced into the reactors at the same level (1×10^7 CFU/ml). For all trials each reactor was placed on a stir plate (Isotemp™ stirrer, Fisher Scientific) set to an agitation level of 150 rpm and incubated at 20 °C. This temperature is higher than the temperature recommended for processing beef products (10 °C) but was chosen to allow better growth of lactic acid bacteria within the time frame of the designed regimen (12 days). The reactors were incubated for 2 h to enable the attachment of cells to the test surfaces, then drained and refilled twice with sterile deionized water to remove loosely associated cells. To simulate the meat processing conditions, reactors were then filled with MS + to just below the overflow side port (~350 ml when all coupon holder are in place), incubated 16 h at 20 °C, again at an agitation rate of 150 rpm to facilitate growth of the bacteria. Following

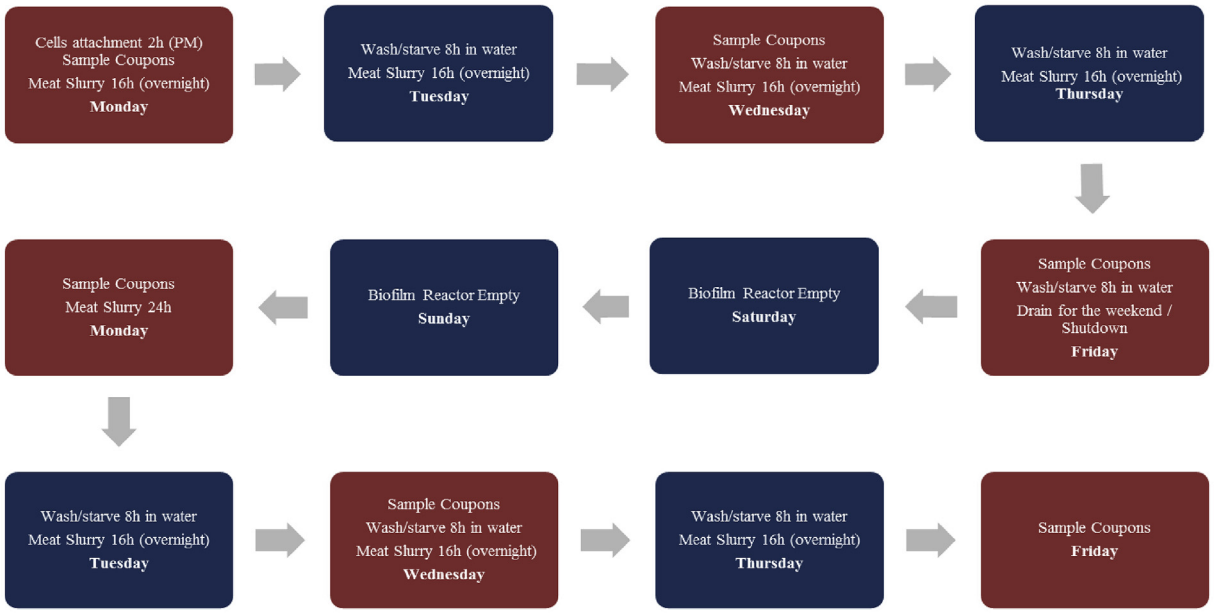

Fig. 1. Schematic diagram of nutrient cycling and sampling plan for the regimen of simulated meat processing conditions (SMPC).

this growth period, the reactors were drained and rinsed twice with sterile deionized water (~350 ml each time) in the same manner as before. The reactors were filled again with sterile deionized water and incubated for 8 h while maintaining the same agitation rate. This succession of water and meat slurry exposure was carried out during the first 5 days of the regimen. On the evening of day-5, and after the second rinse, the reactors were completely drained and left empty for the weekend to imitate a temporary shutdown. On the Monday morning (day-8) following the weekend shutdown, the reactors were filled with sterile MS+ and incubated for 24 h. The following morning, the spent media was drained from the reactors, which were in turn rinsed and refilled with sterile deionized water. This alternation of starvation and nutrient cycles resumed for 4 days. A schematic diagram of the SMPC is presented in Fig. 1.

2.5. Sampling plan

This sampling plan was carried out for all trials with each bacterial strain in mono- or triple species cultures. Every morning, prior to the rinsing step, a sample was aseptically removed from each reactor in order to follow planktonic cell populations. Biofilm samples were taken on Monday, Wednesday and Friday mornings. On these days, one rod containing three coupons of a test surface were taken under sterile conditions to follow biofilm development on the coupons as illustrated in Fig. 1. After the removal of the sample rods, sterile blank rods were aseptically inserted into the ports from which the sample rods had been taken in order to maintain a consistent volume of liquid in the reactors.

2.6. Pre-colonization of coupons by a pioneer strain

An additional set of experiments was designed to examine the effect of the pre-colonization of the coupons by a single bacterial strain on the development of multi-species biofilms. For these experiments each of the three test strains were alternated as the pioneer bacterium. Pre-colonization was carried out in the same manner as described in the above trials by first inoculating the initial sterile 0.85% NaCl solution with the pioneer bacterial strain. As in the other experiments, after 2 h the reactors were drained, rinsed twice with sterile deionized water, filled with MS + for 16 h and then incubated at 20 °C to allow the single strain to colonize the coupons. After the rinsing steps followed by the first 8 h starvation period in water (day 2), the reactors were filled with MS+ and the other two strains were each introduced at levels of 1×10^7 CFU/ml. The growth of these mixed culture biofilms was followed using the same regimen conditions as before; however the sampling plan was modified from that described above. Here, samples were taken on Tuesday and Friday during the first week of the regimen, and Monday and Friday during week two. These experiments were performed in triplicate for each species as the pioneer bacterium.

2.7. Microbiological analyses

For each sample period, a set of three coupons for each test surface were unscrewed from their respective rods and quickly dipped three times in sterile deionized water (10 ml) to remove non-adherent cells. Each coupon was transferred into its own 50 ml tube containing 10 ml of a 0.1% (w/v) Bacto peptone (BD) and 0.1% (v/v) Tween 80 (Sigma, Saint-Louis, MO, USA) solution. In order to remove the cells from the test surfaces, the tubes containing the coupons were vortexed for 30 s at maximum speed and then sonicated for 4 min in an ultrasonic water bath (Branson 5510 (Danbury, CT, USA), 40 kHz). This process was repeated a second time followed by a final 30 s vortex. Samples were serially diluted and spread onto either Tryptic Soy Agar (TSA) for enumeration of *P. fluorescens* or MRS containing 0.006% X-Gal (BioShop Canada Inc., Burlington, ON, Canada) for selective enumeration of *Lactobacillus* and *Leuconostoc* strains. On this medium the *L. pseudomesenteroides* strain produces small blue colonies, while colonies of the *L. plantarum* strain are larger and display a greenish hue. All plates were incubated at 30 °C for 48 h prior to colony counting. The mean of the counts from three coupons was determined. The bacterial density of population per coupon was calculated as described in the Standard Test Method E-2562-07 (ASTM, 2007) and expressed as log CFU/cm^2.

Microbial counts for suspended cells in the bulk meat slurries (planktonic cells) were reported as $\log_{10}$ CFU/ml. Planktonic growth was used to confirm that the MS + supported bacterial growth if coupons displayed poor adherence of cells or were not present. Three independent trials in triplicate were carried out for each treatment and the data presented are the means of these three assays. Each reactor contained 24 coupons for each assay of which 18 were used.

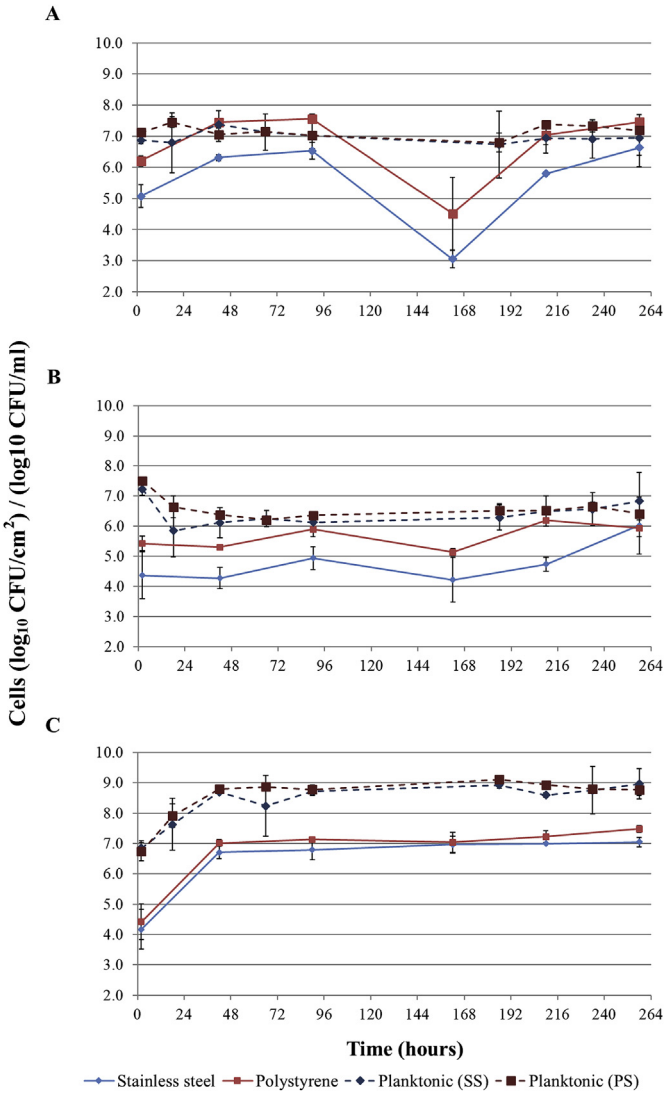

Fig. 2. Planktonic cell counts (log 10 CFU/ml) and populations of (**A**) *L. pseudomesenteroides*, (**B**) *L. plantarum*, and (**C**) *P. fluorescens* attached ($\log_{10}$ CFU/cm^2) to stainless steel or polystyrene coupons in single-species biofilms formed under SMPC. *Error bars are expressed as mean ± SD with n = 9.*

2.8. Statistical analysis

Three independent experimental biofilm reactor trials were performed for each strain on the two surfaces in monoculture as well as with the tri-species combination. For the pre-colonization trials, experiments were also performed in triplicate using each bacterial species as the pioneer strain. Results from these assays were averaged (coupons per time point; n = 9) and statistical analyses (ANOVA) were then performed comparing cells densities of the strains across surface types at each specific sample time during the regimen using GraphPad InStat version 3.10 for Windows (GraphPad Software, San Diego California USA). A value of $P < 0.05$ was used to indicate significant differences.

3. Results

3.1. Planktonic cells in pure cultures

The density of planktonic cells in the bulk MS + medium was not affected by the type of coupons used (Fig. 2). When in pure culture, the level of viable planktonic cells for *L. pseudomesenteroides* remained constant throughout the SMPC. This trend was also observed after 90 h of SMPC, for the two other bacterial test strains. However, during the first 48 h the counts initially decreased for *L. plantarum* while they increased for *P. fluorescens*. The pseudomonad displayed the highest planktonic cell density levels reaching 9.0 $\log_{10}$ CFU/ml after the population stabilized. The L. *pseudomesenteroides* strain maintained the next highest level of planktonic growth at 7.0–7.5 $\log_{10}$ CFU/ml, while planktonic cell densities for *L. plantarum* stabilized approximately 1 log unit lower.

3.2. Biofilm formation by bacterial strains in monoculture

All three of the chosen test strains were able to attach to and/or form single species biofilms on both SS and PS coupons (Fig. 2). Although all bacterial strains were inoculated at the same level (7 $\log_{10}$ CFU/ml), the densities of viable biofilm cell population were found to be both strain and surface dependent for Lactic Acid Bacteria (LAB) while *P. fluorescens* showed similar viable cell coverage for biofilms grown on SS and PS coupons. For LAB, the average density of viable cells detached from surfaces was almost 1 $\log_{10}$ CFU/cm^2 higher on the PS coupons as compared to those obtained from stainless steel during the first 210 h of the SMPC process; however, this difference appeared to lessen by the last day for *L. plantarum*. Moreover, following the initial attachment period (i.e. after 2 h exposure to the inoculated saline) cell population density for *L. pseudomesenteroides* was more than 1 log higher than for either of the other two strains, while the lowest level of cells detached and enumerated following the initial attachment was observed for *P. fluorescens*. At this time, all conditions gave significantly different cell counts ($P < 0.05$) for the LAB with the exception being *L. pseudomesenteroides* on SS compared to *L. plantarum* on PS. Pertaining to all strains, the population density of viable cells collected from the SS coupons increased during the first 90 h of the SMPC process prior to the weekend shutdown. However, the progression of increasing cell numbers differed for each strain but not between surfaces for a given strain. For L. *pseudomesenteroides* viable cell population densities after 42 h on either surface increased by ~1.5 $\log_{10}$ CFU/cm^2 over initial attachment levels and continued an upward trend, albeit at a much slower rate, to 90 h (Fig. 2A). In contrast, the population of viable cells of *L. plantarum* strain displayed no increase by 42 h but samples taken at 90 h showed an increase of 0.6 $\log_{10}$ CFU/cm^2 (Fig. 2B). During this period, the biofilm formation was significantly different between the two LAB on both surfaces ($P < 0.05$). *Pseudomonas fluorescens* had the fastest rate of biofilm growth following initial attachment as cell coverage escalated by almost 3 log units to reach levels of approximately 7.0 $\log_{10}$ CFU/cm^2 during the first 48 h of the regimen; however, these population densities appeared to plateau at this level during the next 48 h (Fig. 2C). The desiccation stress initiated by the simulated temporary shutdown over the weekend had no significant effect ($P > 0.05$) on the cell coverage of *P. fluorescens* as levels after 162 h were the same as those found after 90 h; however, for the LAB the shutdown period caused 1 and 3 log decreases in the populations of harvested adherent viable cells on both surfaces for *L. plantarum* and *L. pseudomesenteroides*, respectively. The cell counts for *L. pseudomesenteroides* on SS were found to be significantly lower ($P < 0.05$) than all others regarding LAB after the shutdown. Nevertheless, following the addition of fresh MS + to the reactors, cell counts for the LAB on the coupons after 210 h increased to reach the respective values that had been observed prior to the simulated shutdown.

Finally, an important detail to note was the growth rates of LAB attached to coupon surfaces immediately following the weekend starvation/desiccation period, where the temporary shutdown seemed to stimulate growth in both LAB strains (Fig. 2A and B). For the L. *pseudomesenteroides* strain, a 1.2 $\log_{10}$ CFU/cm^2 increase in surface associated cells was observed until 42 h, but the increase in growth was more than doubled at 2.6 $\log_{10}$ CFU/cm^2 between 162 and 210 h. Although not as pronounced, the *L. plantarum* strain also showed post-desiccation/starvation stimulation as no growth was observed between

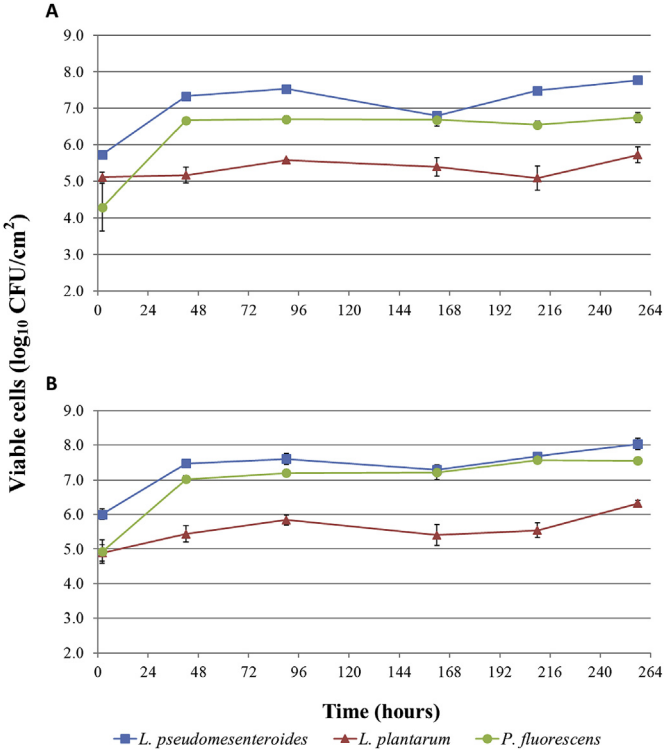

Fig. 3. Viable cell counts for *L. plantarum*, *L. pseudomesenteroides* and *P. fluorescens* in biofilms grown on (**A**) stainless steel or (**B**) polystyrene coupons during SMPC using a mixture of all three strains as inoculum. *Error bars are expressed as mean ± SD with n = 9.*

until 42 h, whereas a 1 log CFU/cm^2 increase was displayed between 162 and 210 h. The cell count was significantly higher ($P < 0.05$) for *L. pseudomesenteroides* on PS after 258 h of growth while the other conditions with LAB were not significantly different ($P > 0.05$).

3.3. Synergistic interactions in multi-species biofilms

Multi-species biofilms were first obtained by introducing all three test strains simultaneously (co-inoculation) into the CDC reactors. Following the initial 2 h attachment period, cell densities for both *Leuconostoc* (5.8 $\log_{10}$ CFU/cm^2) and *Lactobacillus* (5 $\log_{10}$ CFU/cm^2) strains on SS coupons were higher than those observed for the single species biofilms (Figs. 2 and 3), albeit this increase was not significantly higher ($P > 0.05$) for *Lactobacillus*. The number of viable pseudomonad cells harvested after the attachment period when introduced as part of triple species co-culture were virtually identical (4.1 $\log_{10}$ CFU/cm^2) to those obtained when it was introduced alone. In contrast to their monoculture behavior (Fig. 2) the type of coupons had no effect on the initial attachment of the tested LAB strains when administered in the co-culture suspension (Fig. 3). The opposite effect was observed for *P. fluorescens* where no differences were found between initial attachment numbers for the two surface types during the pure culture trials, while in co-culture with the LAB the initial population of adherent *Pseudomonas* cells was higher on PS coupons than on SS ($P < 0.05$). As previously observed in the monocultures, adherent cell populations of all species increased in a similar fashion over the first 90 h of the regimen; however, after this point they followed substantially different trends. In contrast to the results of the monoculture trials, where a dramatic decrease in cell coverage (~3 $\log_{10}$ CFU/cm^2, regardless of surface type) was observed for the *Leuconostoc* strain (Fig. 2), the co-culture appeared to provide a protective capacity as *Leuconostoc* population densities were significantly higher ($P < 0.05$) as they were reduced by only 1 and 0.4 $\log_{10}$ CFU/cm^2 on SS and PS, respectively (Fig. 3). The viable cell population densities in the biofilms for both the *Lactobacillus* and *Pseudomonas* strains appeared to follow a similar trend to that of their monoculture counterparts and remained relatively stable throughout the entire process. *Leuconostoc* was the most abundant bacterium in the population as its viable cell numbers increased significantly ($P < 0.05$) in comparison to those in pure culture, reaching 7.8 and 8.0 $\log_{10}$ CFU/cm^2 on SS and PS, respectively by the end of the SMPC process. Although the levels of the pseudomonad closely mirrored these values on the PS surface, its population densities were 1 $\log_{10}$ CFU/cm^2 lower when SS was employed as the substratum. As for L. *plantarum*, it was found to produce the lowest number of adherent cells throughout all regimens whether in mono or co-culture. Moreover, no significant differences ($P > 0.05$) in cell densities were observed for this bacteria at the end of each regimen, irrespective of surface type or culture condition. Final cell densities on these coupons consistently remained at ~6 $\log_{10}$ CFU/cm^2.

3.4. Pre-colonization

An additional set of experiments was conducted to examine the impact of pre-colonization of the coupon surfaces with one bacterial strain prior to the introduction of the other two strains. In general, the populations of biofilm viable cells by day 12 of the respective regimens were consistently higher on the PS surface (data not shown) but these differences were not statistically significant ($P > 0.05$). The profiles of biofilm development obtained by pre-colonizing both surfaces were completely similar and therefore, only the results on SS are presented herein (Fig. 4). Moreover, the pre-colonization displayed similar trends to those obtained with simultaneous inoculation (Fig. 3). The size and distribution of the different strain populations in the biofilms appeared to be independent of the inoculation sequence (simultaneous versus pre-colonization) and type of surface.

4. Discussion

The bacterial strains used in this study, *Leuconostoc pseudomesenteroides*, *Lactobacillus plantarum* and *Pseudomonas fluorescens* were selected because they represent common spoilage bacteria found on meat products (Borch et al., 1996). *Pseudomonas* spp. are well-known psychrotrophs that rapidly use glucose and degrade amino acids in refrigerated meat products stored under aerobic conditions. Lactic acid bacteria (LAB) such as *Lactobacillus* and *Leuconostoc* are important spoilers of a wide range of food products including cold-stored modified atmosphere packaged cooked meat products (Ercolini et al., 2006). Moreover, it has been demonstrated that these bacteria produce biofilms which are thought to be an important mechanism for their dissemination in food processing settings (Hinsa et al., 2003, Fernandez Ramirez et al., 2015; Padilla-Frausto et al., 2015).

In the present study, the cells of all three bacterial test strains attached readily to both SS and PS coupons, despite the physical stress of the rinsing steps carried out twice a day during the SPMC process (Fig. 2). However, the extent of initial cell adhesion to the coupon surfaces varied with the bacterial strain employed. In the case of both the *Lactobacillus* and *Leuconostoc* strains in monoculture, the population density of viable adherent cells was significantly higher ($P < 0.05$) on PS coupons than on SS. This could in part be due to the different surface properties of the two coupon materials. Indeed, metallic surfaces are usually hydrophilic in nature while polymers such as PS are generally hydrophobic (Pawar et al., 2005) and it is known that bacterial counts from biofilm formation on hydrophobic surfaces are generally higher than on hydrophilic substrata (Pagedar et al., 2010; Di Ciccio et al., 2015; Moreira et al., 2015). In contrast, no differences ($P > 0.05$) were observed for the number of attached cells of *P. fluorescens* on these surfaces and these values were generally the lowest of the three strains tested. This is likely attributed to the fact that the surface properties of the test bacteria would be quite different as *Lactobacillus* and

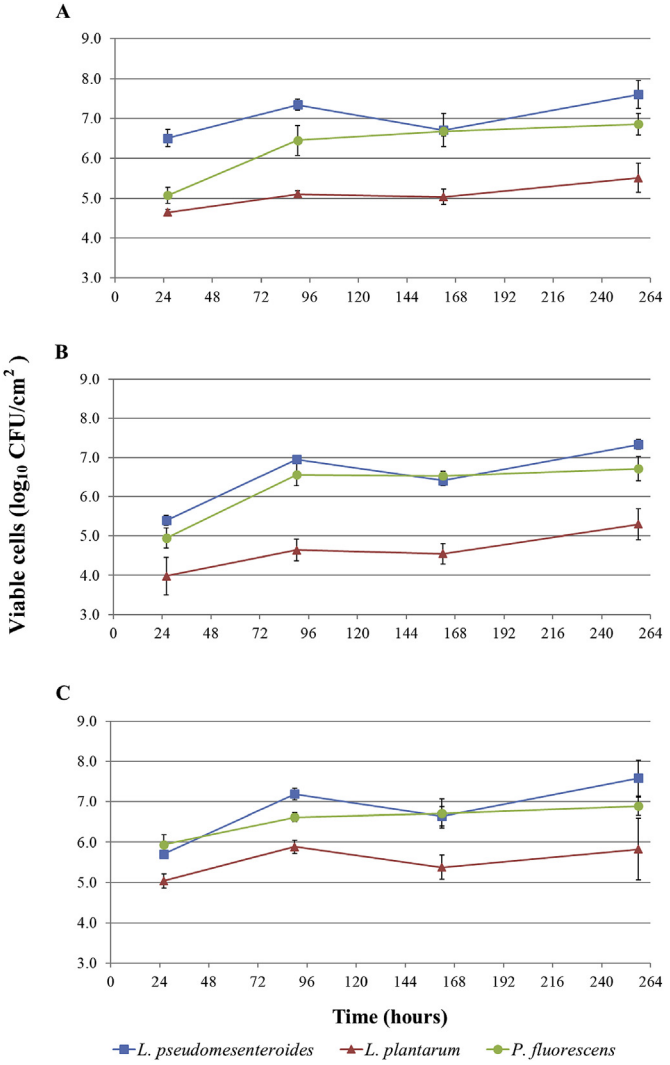

Fig. 4. Populations of viable biofilm cells attached to stainless steel coupons during SMPC following a pre-colonization of the coupons by **(A)** *L. pseudomesenteroides*, **(B)** *L. plantarum* and **(C)** *P. fluorescens*. *Error bars are expressed as mean ± SD with n = 9.*

Leuconostoc are Gram-positive bacteria and *Pseudomonas* is Gram-negative. Thus, the physicochemical properties of the bacterial cell surface, such as the hydrophobicity of the cells, have an important impact on the adhesion during the initial attachment phase, increasing the tendency of microorganisms to adhere to a surface (Van Loosdrecht et al., 1990; Krasowska and Sigler, 2014; Moreira et al., 2015).

Once bacterial cells have attached to a substratum, the process of biofilm maturation begins and overall density and complexity of the biofilm increases due to the active replication of surface-bound cells as well as through recruitment of planktonic cells from the bulk medium (Dunne, 2002). Relative to the other strains examined in the present study, viable cell numbers for *L. pseudomesenteroides* on PS surfaces were significantly ($P < 0.05$) greater than those achieved by the other test strains on either test surface. This same trend was not observed for this bacterium on the SS coupons as CFU/cm^2 values were consistently ~1 $\log_{10}$ CFU/cm^2 lower than those on PS during initial attachment and for all sample times throughout the regimen. The L. *plantarum* strain was the weakest surface colonizer; however, it showed the same tendency as the *L. pseudomesenteroides* strain in terms of surface preference. This underscores the importance of the initial attachment stage for these Gram-positive species towards their overall success in developing mature biofilms under our test conditions (Palmer et al., 2007).

Kubota et al. (2008) observed that *L. plantarum* formed better biofilm than any other LAB they isolated in their study. The contrasting results obtained in the present study may be attributed to the different conditions under which the biofilms were developed. The shear stress produced by the agitation in the reactors may have decreased the ability of *L. plantarum* to form a biofilm resulting in the weakly adherent cells being dispersed in the bulk medium. The dispersion may happen by shedding of daughter cells from actively growing cells, detachment as a result of quorum sensing or shearing of biofilm aggregates because of flow effects (Donlan, 2002). Also it not be excluded that, for the particular lactobacilli strain used (ATCC 10241), the MS+ was probably not sufficient for supplying these cells with the required nutrients supporting their EPS production. Conversely, although the *P. fluorescens* strain had the lowest initial attachment levels of the three test bacteria, this did not influence its ability to rapidly form mature biofilms as viable cell numbers on both surfaces increasing by 3 $\log_{10}$ CFU/cm^2 from initial attachment levels and reaching maximum density levels after only 42 h. This contrast in behaviours between test strains is believed to be due to, at least in part, superior extracellular polymeric substances (EPS) production by the pseudomonad (Sasahara and Zottola, 1993; Simões et al., 2007). The role of EPS in the recruitment of and retention of cells within the biofilm matrix has been demonstrated elsewhere (Habimana and Casey, 2018). Unfortunately, we did not quantitate EPS levels in these experiments to corroborate this theory.

The populations of adherent cells for all three test strains on either coupon surface increased above the initial attachment levels until 90 h into the SMPC process, at which time the reactors were drained and remained empty over the weekend. This resulted in significant decreases ($P < 0.05$) in surface associated viable cells for the two LAB strains but not *P. fluorescens*. The L. *pseudomesenteroides* strain, irrespective of surface type was most affected (~3 $\log_{10}$ CFU/cm^2 decrease) and while reductions in CFU/cm^2 for *L. plantarum* were ~1 $\log_{10}$ CFU/cm^2. These losses could be due to a die-back of the cells in response to nutrient deprivation and desiccation stress encountered during the simulated shutdown. Moreover, depletion of nutrients could also cause the detachment of cells from surfaces when the coupons were dipped in water prior to processing (Hunt et al., 2004; Rochex and Lebeault, 2007). Despite the reductions of adherent viable cells for the two LAB strains, by the end of the SMPC process (258 h) their numbers had recovered to equal or exceed the levels achieved just prior to the shutdown period. Once rehydrated, continued biofilm growth would be initiated on the surface itself and through the shedding of the persistent cells into the bulk medium, resulting in substantial increases in planktonic cell numbers which in turn could be recruited back to the established biofilm on the surface (Donlan, 2002). The perceived higher rate surface colonization for these bacteria after the shutdown, relative to that observed at the onset of the SMPC regimen (Fig. 2), is likely due to major differences in surface compositions. At the start of the regimen, bacteria were exposed to pristine surfaces. Since *Pseudomonas* spp. are known to be good producers of EPS this could explain the fact that the same shutdown conditions had no effect on viable cell counts for this strain (Sasahara and Zottola, 1993). A plausible reason for this result could be related to the entrapment/binding of water within EPS layers, thereby helping to prevent the negative consequences of desiccation (Hansen and Vogel, 2011). A thin film at the air-liquid interface was present on the inner surfaces of the reactor vessel on the third day of the SPMC process for trials involving the *P. fluorescens* strain. This observation is in agreement with previous work reporting the capability of *P. fluorescens* to form surface-attached biofilms and floating biofilms at air-liquid interfaces (Koza et al., 2009; Ueda and Saneoka, 2015). The presence of a pellicle type biofilm at the air-liquid interface for this culture also suggests that some components of its EPS are amphiphilic. Exopolysaccharides, particularly cellulose, have been identified as major components of the pellicles of *Pseudomonas* (Armitano et al., 2014). In the current study, the associated

hydrophobic segments may have contributed to preventing evaporation of water present within the matrix which consequently allowed the *Pseudomonas* strain to resist the desiccation stress. The relative hydrophobicity of its matrix could also have reduced the extraction of water soluble nutrients following the rinsing steps preceding the shutdown but this was not verified in the present study.

The co-culture experiments revealed a potential synergistic behavior between strains which may explain the relative higher initial attachment values (an increase of almost 1 $\log_{10}$ CFU/cm^2) for *Leuconostoc* and *Lactobacillus* strains on SS coupons when all three test strains were added simultaneously to the reactors (Fig. 3) as compared to their incorporation as single species (Fig. 2). The presence of one species colonizing a coupon surface could potentially provide ligands that enhance attachment of the other bacterium as it moves within close proximity to the surface (Leung et al., 1998) or instigate interactions between cells already at the surface to stimulate the formation of biofilms (Branda et al., 2005). Moreover, EPS produced by one bacterial strain, such as *P. fluorescens*, may help entrap other bacteria and facilitate their attachment while also assisting in the sequestering of nutrients and desiccation protection during the shutdown. This potential effect for the EPS produced by *Pseudomonas* could explain the smaller viability losses of the *Leuconostoc* strain observed after 162 h in the multispecies biofilms (1 $\log_{10}$ CFU/cm^2, Fig. 3) relative to their single species counterparts (~3 $\log_{10}$ CFU/cm^2, Fig. 2A). Pre-colonization with *P. fluorescens* or *L. pseudomesenteroides* cells resulted in an increase in the adherent cell populations for *L. plantarum*, supporting the theory of a synergistic effect between the different species. This observation is in agreement with other studies reporting that the presence of other microorganisms on specific surfaces, such as glass and polyurethane conveyor belts, increasing the attachment of co-cultivated strains (Castonguay et al., 2006; Klayman et al., 2009a; Marouani-Gadri et al., 2009). The synergism could include presence of favorable attachment factors in neighbor strains (co-aggregation) and production of nutrients of interest (metabolic cooperation) (Elias and Banin, 2012). Cell-to-cell signaling by means of auto-inducer chemicals and so-called quorum sensing (Giaouris et al., 2015) has also been demonstrated to play a role in cell attachment to surfaces (Donlan, 2002). However, other factors contributing to the observed differences in attachment should be noted. In particular, the presence of a conditioning film on coupon surfaces would likely have occurred since the pioneer bacterium was allowed to grow for 16 h in MS + before the introduction of the other strains. Organic molecules present in the meat would most certainly coat the coupons thereby altering the physicochemical properties of the interface (McEldowney and Fletcher, 1987).

5. Conclusion

This work provides useful information on single and multi-species biofilm ecology under conditions simulating those encountered in meat processing industries. Our results showed that the attachment of the two Gram-positive strains studied here was more pronounced on a hydrophobic surface (PS) as compared to that observed on a hydrophilic test surface (SS); however, the Gram-negative test strain, *Pseudomonas fluorescens*, was not affected by the nature of the surfaces. The results also provided evidence of a synergistic effect between *P. fluorescens, L. plantarum* and *L. pseudomesenteroides* in multi-species biofilms. The attachment of the *Lactobacillus* strain was improved by 1 log CFU/cm^2 following a pre-colonization of the surface by the Gram-negative strain. It was also observed that the presence of that particular strain significantly enhanced the resistance of *Leuconostoc* in absence of a liquid environment. It was therefore suggested that the amphiphilic nature of some EPS of the *Pseudomonas* matrix could be involved in such an effect.

Finally, the simulated meat processing conditions (SMPC) model developed in this study can serve as a baseline that can be build upon in future work. Regimen parameters (temperature, nutrient supply, combinations of microorganisms, etc.) can be systematically altered to emulate specific processing environments and the overall complexity may be increased through appropriate experimental design. Therefore, this could have utility in competition studies between food-borne pathogens and non-pathogenic bacteria during biofilm development. It may also serve as a template to study the impact of cleaning agents on multi-species biofilms harbouring strains typically found in meat processing industries or other food processes. Experiments are underway to study EPS production during single and multi-species biofilms formation by the bacterial strains used in this study.

Therefore, following the results obtained in this study, we can suggest that certain non-pathogenic strains such as Lactic Acid Bacteria could be selected and used to form a precompetitive biofilm which can act as a barrier to the growth of food-borne pathogens in biofilms if we consider that any food processing/food equipment surfaces has to be colonized by bacteria.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.022.

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