Interactions between *L. monocytogenes* and *P. fluorescens* in Dual-Species Biofilms under Simulated Dairy Processing Conditions †

Francesca Maggio 1, Chiara Rossi 1, Clemencia Chaves-López 1, Annalisa Serio 1, Luca Valbonetti 1, Francesco Pomilio 2, Alessio Pio Chiavaroli 1 and Antonello Paparella 1, *

1 Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Via R. Balzarini 1, 64100 Teramo, Italy; fmaggio@unite.it (F.M.); crossi@unite.it (C.R.); cchaveslopez@unite.it (C.C.-L.); aserio@unite.it (A.S.); lvalbonetti@unite.it (L.V.); alessio.chiavaroli27@gmail.com (A.P.C.)

2 Food Hygiene Unit, NRL for *L. monocytogenes*, Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise “G. Caporale”, 64100 Teramo, Italy; f.pomilio@izs.it

* Correspondence: apaparella@unite.it; Tel.: +39-0861-266944

† Presented at the 1st International Electronic Conference on Food Science and Functional Foods, 10–25 November 2020; Available online: https://foods_2020.sciforum.net/.

Abstract: In dairy processing environments, many bacterial species form biofilms on surfaces and equipment. Among them, *Listeria monocytogenes* and *Pseudomonas* spp. could be present in mixed-species biofilms with increased resistance to disinfectants. This study aimed to evaluate the interactions between *L. monocytogenes* and *P. fluorescens* in dual-species biofilms simulating dairy processing conditions as well as the capability of *P. fluorescens* to produce the blue pigment. The biofilm-forming capability of single- and mixed-cultures was evaluated on polystyrene (PS) and stainless steel (SS) surfaces at 12 °C for 168 h. Biofilm biomass was assessed by crystal violet staining, the planktonic and sessile cells were quantified in terms of Colony Forming Unit (CFU), and the carbohydrates were quantified by the anthrone method. The biofilms were also observed through Confocal Laser Scanning Microscopy (CLSM) analysis. Results showed that only *P. fluorescens* was able to form biofilms on PS. In dual-species biofilms at the end of the incubation time, a lower biomass compared to *P. fluorescens* mono-species was observed. On the SS, the biofilm cell population of *L. monocytogenes* was higher in the dual-species than in the mono-species, particularly after 48 h. Carbohydrates in the dual-species system were higher than those of the mono-species and were also revealed at 168 h. The production of blue pigment by *P. fluorescens* in the Ricotta-based model system was revealed in both single cultures and co-cultures and was confirmed by the CLSM results, showing agglomeration, probably linked to the blue pigment. Our study suggests that the interactions between the two species can influence biofilm formation, but not the capability of *P. fluorescens* to produce blue pigment.

Keywords: *Listeria monocytogenes*; *Pseudomonas fluorescens*; multi-species; biofilms; blue pigment; dairy product

1. Introduction

Microbial biofilms are three-dimensional structures of various bacteria that adhere to biotic or abiotic surfaces and differentiate into complex communities, embedded within extracellular polymeric substances (EPS) [1]. The relevance of microbial biofilms has been described in different fields including the food industry where biofilms are responsible for potential food contamination, corrosion, and economic losses [2]. Particularly in the dairy industry, many bacterial species adhere to and form biofilms on surfaces and equipment. Among them, *Listeria monocytogenes* and different species of *Pseudomonas* [3] are
worthy of attention. *Listeria monocytogenes* is a ubiquitous pathogen, able to colonize, and persist on common surfaces in food processing environments [4]; it can also contaminate a wide variety of foods such as dairy products. The microbiota of refrigerated foods is dominated by selected microorganisms, including *Pseudomonas* spp. In particular, *P. fluorescens* can form biofilms on the surfaces of dairy processing plants [5] and produce pigments such as pyoverdine, pyocyanin, and indigoidine, which are responsible for dairy products’ blue discolouration [6].

Biofilms found in nature are generally formed by two or more microbial species; in fact, multi-species biofilms are commonly encountered in food and food-related environments [7]. Multi-species biofilms possess properties unavailable in mono-species biofilms, which may provide advantages to microorganisms such as an increase in tolerance against stressful conditions and the capability to degrade organic compounds [8].

The objective of this study was to evaluate dual-species biofilms formed by *L. monocytogenes* and *P. fluorescens* in a system simulating real conditions encountered in dairy processing. This was done by using (i) surfaces of PS and SS; (ii) *L. monocytogenes* and blue-pigmenting *P. fluorescens* strains, isolated from dairy products; (iii) a Ricotta-based model system as the growth medium; and (iv) 12 °C as the incubation temperature.

2. Materials and Methods

Eight strains of *Listeria monocytogenes* were tested together with one strain of blue-pigmenting *Pseudomonas fluorescens* pf5 isolated from mozzarella cheese [5]. *L. monocytogenes* strains, previously isolated from dairy products (LM 1-2-3-4) and dairy plants (LM 5-6-7-8), were typed and serotyped according to the Pulsed-Field Gel Electrophoresis (PFGE) and the US/FDA Bacteriological Analytical Manual [9]. To simulate a dairy processing environment, a Ricotta-based model system was prepared following the method described by de Carvalho et al. (2015) [10].

In order to examine the biofilm-forming capability of the strains in mono- and dual-species and to select one combination, the biofilm study was first assessed on PS. The bacterial suspensions (10⁵ CFU/mL), prepared in the Ricotta-based model system, were aliquoted on PS microtitre plates and then incubated at 12 °C for 168 h. The total biomass was quantified at 590 nm by crystal violet assay [5].

To evaluate biofilm formation on stainless steel (AISI 304 coupons), *L. monocytogenes* LM5 strain was chosen and combined with *P. fluorescens* pf5. Sterile glass containers with coupons and the inoculated Ricotta-based model system were incubated at 12 °C for 168 h. At 0, 48, 72, 96, and 168 h, planktonic cells were enumerated on selective media for *Pseudomonas* spp. (*Pseudomonas* agar base) and for *L. monocytogenes* (Agar Listeria according to Ottaviani & Agosti). The enumeration of cells in biofilms (sessile cells) was performed by scraping the surfaces with cotton swabs to collect and enumerate the cells [11].

The EPS were extracted as previously reported by Abdallah et al. (2015) [12], with some modifications, while carbohydrates quantification was carried out following the anthrone method [13]. The absorbance of the samples at 625 nm was measured and the results were presented in µg/cm². Mono- and dual-species biofilm structures were observed by CLSM according to the method described by Rossi et al. (2018) [2]. The biofilms were stained with the LIVE/DEAD BacLight Bacterial Viability kit. The fluorescence of pyoverdine, the siderophore produced by *P. fluorescens*, was also checked [14]. Data of biofilm assays were subjected to analysis of variance (ANOVA) and pair-comparison within the same group was achieved by applying Dunnett’s test procedure at *p* < 0.05 using XLSTAT ver. 2017 (Addinsoft, Paris, France).

3. Results and Discussion

3.1. Serotype and Pulsotype of *L. monocytogenes* Strains

Four serotypes (1/2a, 1/2b, 1/2c, and 4b) were identified among the eight *L. monocytogenes* strains. The most prevalent serotype was 1/2b (for strains isolated from both food
and environmental sources), then 1/2a (for food strains) and 1/2c (for environmental strains), followed by 4b (for the mozzarella cheese isolate). A total of eight ApaI and eight AscI PFGE types were distinguished, thus revealing that the strains isolated from food products and the environment were genetically different and heterogeneous.

3.2. Biofilm Formation on the Polystyrene Surface

None of the eight *L. monocytogenes* strains were able to form biofilms on PS (data not shown). However, numerous studies have demonstrated that this pathogen is able to form biofilms on various surfaces [15,16], and a previous study reported low Optical Density (OD) values for *L. monocytogenes* biofilms [17]. On the other hand, *P. fluorescens* exhibited good biofilm formation capacity, with increased biomass during incubation time. However, a different behaviour was observed for the species in combination, with variability among the strains. At the end of the incubation period, biofilms in dual-species systems were significantly lower than the single ones (*p < 0.05), and a higher biofilm biomass for the combinations *P. fluorescens*—*L. monocytogenes* LM5 was noticed after 72 h. With respect to the blue discolouration, *P. fluorescens* blue pigment production was observed both in single and mixed cultures after 72 h. Based on the obtained results, the *L. monocytogenes* LM5 and *P. fluorescens* pf5 combination was selected for subsequent analysis.

3.3. Biofilm Formation on the Stainless Steel Surface and Enumeration of Planktonic and Sessile Cells

The results of *L. monocytogenes* LM5 and *P. fluorescens* pf5 planktonic and sessile cells on SS coupons are presented in Figure 1.

Regarding the planktonic phenotype (data not shown), the presence of *P. fluorescens* determined a slight decrease of *L. monocytogenes* counts at 48 and 96 h. *P. fluorescens* showed a greater increase in load over time compared to *L. monocytogenes*, and planktonic counts did not significantly differ between mono- and dual-species.

The results regarding the sessile populations (Figure 1) showed that *L. monocytogenes* was able to adhere on the SS surface, probably because of the affinity established between the charge of the cell surface and the anchoring site. In fact, at low temperatures, *L. monocytogenes* increases the hydrophilicity and, therefore, the affinity to hydrophilic surfaces such as steel [18]. The pathogen load in mono-species increased up to 3.27 log CFU/cm² at 72 h with no particular changes during the time. In multi-species conditions, the presence of *P. fluorescens* statistically (*p < 0.05) increased the pathogen biofilms after 48 h when it reached a sessile population of 3.39±0.36 log CFU/cm². This result is in line with those reported by Puга et al. (2018) [17], who observed a stimulation of *L. monocytogenes* adhesion in mixed-culture biofilms with *P. fluorescens*. The authors linked the positive effects on *L. monocytogenes* to *Pseudomonas* production of proteinases able to mobilize essential amino acids. At the end of the experimental time, *L. monocytogenes* LM5 sessile cells in the mixed-culture dropped to 1.4 log CFU/cm². The observed fast cellular dispersal for multi-species biofilms could have been stimulated from the early achievement of a high biofilm level with no extra nutrient supplementation [17]. The *P. fluorescens* sessile population reached 3.58 ± 0.34 log CFU/cm² at 48 h and decreased over time, reaching 1.4 log CFU/cm² after 168 h, with no statistically significant differences among single- and mixed-cultures. The blue pigment production of *P. fluorescens* was observed starting from 72 h both in single- and in mixed-cultures (Figure 2) when the highest load of the spoilage microorganism (planktonic cells) was detected (data not shown). In agreement with our findings, Andreani et al. (2015) [19] observed an evident blue pigment in broth when *Pseudomonas* counts reached about 7 × 10⁷ CFU/mL and concluded that the blue pigment production took place in the late logarithmic phase.
Figure 1. Dynamics of sessile cells of *L. monocytogenes* LM5 and *P. fluorescens* pf5 in mono- and dual-species conditions on stainless steel (SS) coupons at 12 °C for 168 h. The asterisk (*) means statistically significant difference between the mono- and dual-species of each strains for the same incubation time (* p < 0.05). L: *L. monocytogenes* in single-species; L + P: *L. monocytogenes* in dual-species; P: *P. fluorescens* in single-species; P + L: *P. fluorescens* in dual-species.

Figure 2. (A) Blue pigment colour appearance during the evaluation of biofilm formation on SS coupons in glass containers. From the left: control, *P. fluorescens* pf5, *L. monocytogenes* LM5, and the dual-species system. (B) Blue pigment colour evolution during the CLSM analysis: (B1) 48 h, (B2) 72 h, (B3) 96 h, and (B4) 168 h.

3.4. EPS Analysis by Carbohydrates Quantification

The total amount of carbohydrates in the biofilms was affected by the time and the species involved in biofilm formation (Figure 3). The biofilm carbohydrates content increased over time in single-species biofilms, with the greatest increase occurring at 96 h. No carbohydrates were revealed at 168 h for both single-species biofilms. In dual-species conditions, a higher yield in carbohydrates in comparison with the single species was detected at 72 h. Remarkably, the carbohydrates of the dual-species biofilms were also revealed at the end of the experimental time. Puiga et al. (2018) [17] reported that the inclusion of *L. monocytogenes* to the already established *P. fluorescens* biofilms increased matrix production.
Figure 3. Carbohydrates amount (µg/cm²) from *L. monocytogenes* LM5 and *P. fluorescens* pf5 biofilms on SS coupons in mono- and dual-species at 12 °C for 168 h. The asterisk (*) means statistically significant difference between the mono- and dual-species of each strains for the same incubation time (* p < 0.05). P: *P. fluorescens* in single-species; L: *L. monocytogenes* in single-species; P + L: *P. fluorescens* and *L. monocytogenes* in dual-species.

3.5. Confocal Laser Scanning Microscopy Analysis

As observed from CLSM analysis (Figure 4), no three-dimensional biofilm architecture was revealed at 168 h. In fact, Figure 4 shows green agglomerates containing damaged or dead cells (red cells according to the propidium iodide staining) and detached *P. fluorescens* cells (the blue colour of pyoverdine fluorescence). The fact that the agglomerates were not clearly identifiable as cells, and that they were present only in the samples with *P. fluorescens*, suggests that they could depend on blue pigment formation. In addition, this particular behaviour was observed starting from 72 h in correspondence with the blue pigment formation by *P. fluorescens*. Figure 2a–d revealed that blue pigment discolouration starting from 72 h turned into a green/grey colour. The colour change highlights the possible reduction of indigoidine to leucoindigoidine, which is considered a chemical marker of blue discolouration [20].

Figure 4. Confocal laser scanning microscopy analysis of *L. monocytogenes* LM5 and *P. fluorescens* pf5 biofilms in dual-species conditions after 168 h at 12 °C.

4. Conclusions

Our results showed that the behaviour of planktonic and sessile populations on SS coupons was strongly dependent on the culture conditions (mono-/dual-species). In fact, the presence of *P. fluorescens* increased the *L. monocytogenes* sessile population and the total EPS carbohydrates amount on the SS coupons. Nevertheless, further studies including the comparison of the gene expression between single- and dual- species biofilms and interactions in terms of the volatilome would be useful to provide more information on the inter-species consortium.
Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request.

Conflicts of Interest: The authors declare no conflicts of interest.

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