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

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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 CFU and the carbohydrates were quantified by 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. Moreover, in dual-species biofilms at the end of the incubation time, a lower biomass compared to *P. fluorescens* mono-species was observed. On SS, the biofilm cell population of *L. monocytogenes* was higher in the dual-species than in mono-species, particularly after 48 h. Carbohydrates in dual-species system were higher than those of the mono-species and were revealed also at 168 h. The production of blue pigment by *P. fluorescens* in the Ricotta medium was revealed both in single- and co-culture, confirmed also by CLSM results, showing agglomeration, probably linked to the blue pigment. Our study highlights 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 of potential food contamination, corrosion and economic losses [2]. Particularly in the dairy industry, many bacterial species adhere and form biofilms on surfaces and equipment and, 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 the food processing environments [4], capable also to 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 onto surfaces of the dairy processing plants [5] and produce pigments such as pyoverdine, pyocianin and indigoidine, responsible of 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 the 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, by using (i) surfaces of polystyrene and stainless steel; (ii) *L. monocytogenes* and blue pigmenting *P. fluorescens* strains, isolated from dairy products; (iii) a Ricotta-based dairy model as growth medium, (iv) 12 °C as 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 PFGE and to the US/FDA Bacteriological Analytical Manual [9].

To simulate a dairy processing environment, a Ricotta-based dairy model 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, at first the biofilm study was assessed on PS. The bacterial suspensions (10^5 CFU/mL), prepared in the Ricotta medium, were aliquoted on PS microtitre plates and then incubated at 12 °C for 168 h. 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 inoculated Ricotta medium 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 scraping the surfaces by 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 $\mu g/cm^2$.

Mono- and dual-species biofilm structure was observed by CLSM according to the method described by Rossi et al. (2018) [2]. The biofilms were stained with 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 applying Tukey'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 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 environment were gentically different and heterogeneous.

3.2. Biofilm Formation on Polystyrene Surface

None of the eight *L. monocytogenes* strains was able to form biofilms on PS (data not shown). Although numerous studies have demonstrated that this pathogen is able to form biofilms on various surfaces [15,16], also a previous study reported very low 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. Although at the end of the incubation period, biofilms in dual-species systems were significantly lower than the single ones, 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 culture after 72 h. Based on the obtained results, the combination *L. monocytogenes* LM5 and *P. fluorescens* pf5 was selected for the subsequent analysis.

3.3. Biofilm Formation on 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, respectively. *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 SS surface, probably due to 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/cm^2 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/cm^2. This result is in line with those reported by Puga 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 mixed-culture dropped to 1.4 Log CFU/cm^2. The observed fast cellular dispersal for multi-species biofilms could have been stimulated from the early achievement of high biofilms level with no extra nutrient supplementation [17]. *P. fluorescens* sessile population reached 3.58 ± 0.34 Log CFU/cm^2 at time 48 h and decreased over time reaching 1.4 Log CFU/cm^2 after 168 h, with no statistically significant differences among single and mixed culture. The blue pigment production of *P. fluorescens* was observed starting from 72 h both in single- and in mixed- culture (Figure 2), when the highest load of the spoilage microrganism 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 7×10^8 CFU/mL and concluded that the blue pigment production took place in the late logarithmic phase.

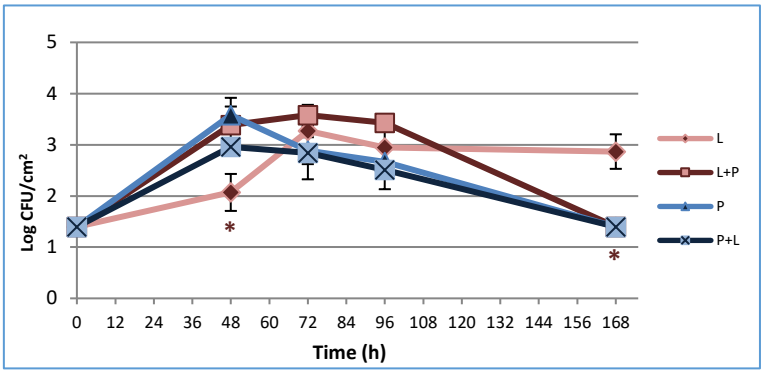

Figure 1. Dinamics of sessile cells of *L. monocytogenes* LM5 and *P. fluorescens* pf5 in mono- and dual-species conditions on SS coupons at 12 °C for 168 h. L: *L. monocytogenes* in single species; L + P: *L. monocytogenes* in dual-species; P: *P. fluorescens* in single species; P + L: *P. fluorescens* in dual-species.

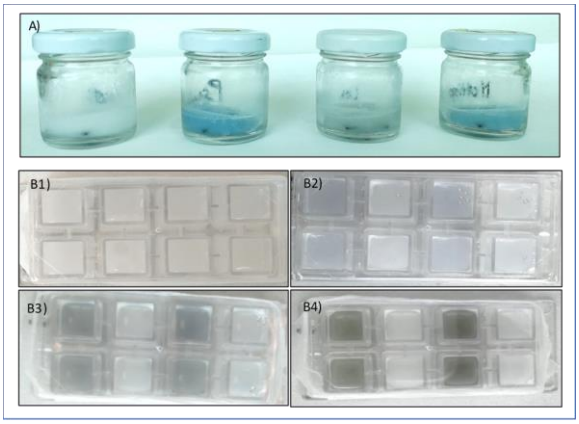

Figure 2. (**A**) Blue pigment colour appearance during the evaluation of biofilm formation on SS coupons in glass containers. From the left: Control, *P. fluorescens* pf5, *L. monocytogenes* LM5 and dual-species system. (**B**) Blue pigment colour evolution during the CLSM analysis: (**B1**) 48 h, (**B2**) 72 h, (**B3**) 96 h, (**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. Instead, 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 revealed also at the end of the experimental time. With this respect, also Puga et al. (2018) [17] reported that the inclusion of *L. monocytogenes* to the already established *P. fluorescens* biofilms increased matrix production.

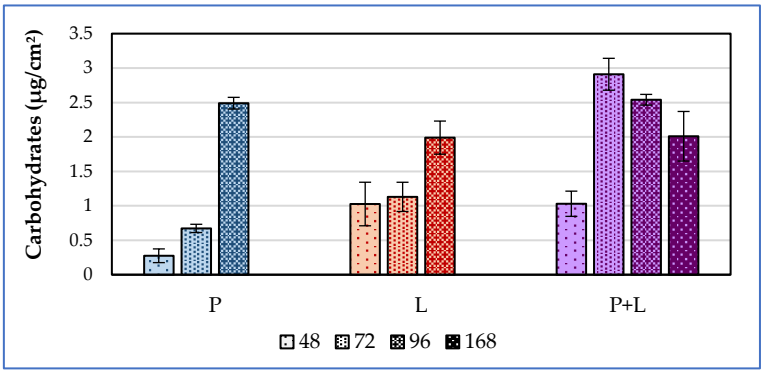

Figure 3. Carbohydrates amount (µg/cm²) from *L. monocytogenes* LM5 and *P. fluorescens* pf5 biofilms on SS coupons in mono- and dual-species at 12 °C for 168 h. P: *P. fluorescens* in single species; L: *L. monocytogenes* in single species; P + L: *P. fluorescens* and *L. monocytogenes* in dual-species.

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 PI staining) and detached *P. fluorescens* cells (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 corrispondence of the blue pigment formation by *P. fluorescens*. The 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].

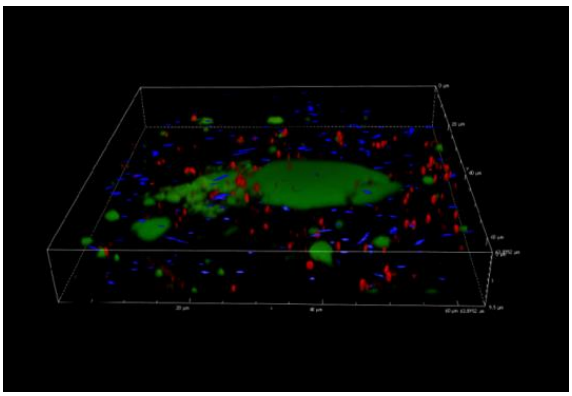

Figure 4. Confocal Laser Scanning Microscopy analysis of *L. monocytogenes* LM5 and *P. fluorescens* pf5 biofilms in dual-species conditions after 168 h at 12 °C.

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 *L. monocytogenes* sessile population and total EPS carbohydrates amount on SS coupons. Nevertheless, more studies including the comparison of the gene expression between single- and dual- species biofilms and interactions in terms of volatilome would be useful to provide more information on the inter-species consortium.

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

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