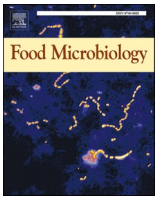
Food Microbiology 105 (2022) 104026

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Dual-species biofilms formation between dominant microbiota isolated from a meat processing industry with *Listeria monocytogenes* and *Salmonella enterica*: Unraveling their ecological interactions

C. Ripolles-Avila, M. Guitan-Santamaria, K. Pizarro-Giménez, T. Mazaheri, J.J. Rodríguez-Jerez *

Human Nutrition and Food Science Area, Departament de Ciència Animal i dels Aliments, Universitat Autònoma de Barcelona (UAB), Edifici V, Campus de la UAB, 08193, Bellaterra (Cerdanyola del Vallès), Barcelona, Spain

ARTICLE INFO

Keywords:
Biofilms
Interactions
Persistence
Biocontrol
L. monocytogenes
Salmonella spp.

ABSTRACT

Alternatives to combat the persistence of pathogens need to consider the microbiota established on industrial surfaces as they can influence the protection or replacement (*i.e.* reduction/inhibition) of pathogens. The objective of the present study was to determine the ecological interactions established in dual-species biofilms between *Listeria monocytogenes* and *Salmonella enterica* as target pathogens, and isolates recovered from a meat processing facility (*i.e. Pseudomonas fluorescens, Pseudomonas fragi, Bacillus safensis, Bacillus megaterium,* and *Candida zeylanoides*). Results showed different ecological relations in biofilms depending on the species evaluated. *Pseudomonas* spp. did not influence the growth of either pathogen, although tested species tended to protect the pathogens in the structures generated. *B. megaterium* and *C. zeylanoides* affected the two pathogens differently, demonstrating a reduction of *L. monocytogenes* adhered cells within the formed biofilm. *B. safensis* reduced or presented non-influence on *S. enterica* depending on the incubation conditions. Contrarily, *B. safensis* was the microorganism that demonstrated the highest replacement capacity for *L. monocytogenes*, reducing its growth by up to 4 log CFU/cm^2. The *in vitro* study of bispecies biofilms is important for the food industry, helping to understand how they behave and to find an effective way to eliminate them.

1. Introduction

Surfaces in the food industry are regularly exposed to cleaning and disinfection processes since the presence of biofilms, the most widespread microbial form in the environment, is constantly detected on them (González-Rivas et al., 2018; Mazaheri et al., 2021). Biofilms are biostructures where microorganisms are embedded in a produced organic matrix essentially composed of polysaccharides, proteins, and nucleic acids (Flemming and Wingender, 2010). This matrix contributes to the attachment of microbial cells to the surface and triggers tolerance to environmental stresses (Álvarez-Ordóñez and Briandet, 2016). Biofilms are difficult to remove completely, becoming a source of contamination for food products due to the continuous shedding of cells and spores from food and non-food contact surfaces (González-Rivas et al., 2018; Ripolles-Avila et al., 2018b). Biofilms provide protection to cells by presenting a physical barrier, induce stress proteins, permit gene exchange, prevent desiccation and could also produce certain substances for protecting the niche against competitive microorganisms (Bridier et al., 2011; Fagerlund et al., 2017). Furthermore, tolerance has been related to the resistance of microbial cells when they are exposed to sub-lethal concentrations of disinfectants (Capita et al., 2014; Molina-González et al., 2014; Rodríguez-Melcón et al., 2019). Regarding cleaning and disinfection procedures, their aim is to reduce the numbers of microorganisms to an acceptable level to manage risk (Ripolles-Avila et al., 2019c). However, there are intrinsic and extrinsic factors such as temperature, water availability, and the presence of organic matter that constantly influence the growth of microorganisms (Fagerlund et al., 2021). Such factors imply that a whole microbial community could be able to survive and remain on different surfaces of the food processing facilities conforming an ecosystem named resident microbiota (Brightwell et al., 2006; Hascoët et al., 2019). It has been indicated that the resident microbiota directly influences (*i.e.* modulate the growth of a microorganism) the presence of certain foodborne pathogens such as *Listeria monocytogenes* (Fagerlund et al., 2021).

A great diversity of resident microbiota, including both microbial families and species capable of adhering to industrial surfaces and

* Corresponding author.
E-mail address: josejuan.rodriguez@uab.cat (J.J. Rodríguez-Jerez).

https://doi.org/10.1016/j.fm.2022.104026
Received 17 November 2021; Received in revised form 8 March 2022; Accepted 11 March 2022
Available online 14 March 2022

developing biofilms, is found in the food industry (Speranza et al., 2017). On industrial surfaces (*i.e.* contact and non-contact food surfaces), species from the *Enterobacteriaceae* family, *Pseudomonas* spp., *Acinetobacter* spp., *Bacillus* spp., *Staphylococcus* spp., lactic acid bacteria (LAB), and certain yeasts generally tend to predominate (Fagerlund et al., 2017; Møretrø and Langsrud, 2017). A previous study from our research group evaluated the resident microbiota on the surfaces of an Iberian pig processing plant and correlated the microbial profiles when *L. monocytogenes* was present or absent from the surfaces (Hascoët et al., 2019). It was demonstrated that abundance in *Pseudomonas* spp. influenced positively *L. monocytogenes* presence and other genera such as *Bacillus* spp. diminished the presence of the pathogen. On this regard, Hascoët et al., (2021) demonstrated that mature preformed *Bacillus safensis* biofilms can inhibit adhesion and the biofilm formation of multiple *L. monocytogenes* strains, eliminating the pathogen by a currently unidentified mechanism. So, resident microbiota in food processing plants can influence the growth of pathogens residing in the food environment (Fagerlund et al., 2021). This is because of biofilms can be formed by single species or by different species. In that last case, interactions between cohabiting species influence their assembly and function, as well as lead to outgoing properties that are specific to multi-species biofilms (Røder et al., 2020). For example, mixed biofilms are more resistant towards distinct compounds such as antimicrobials than monospecies biofilms. This is because they generate interspecies interactions for metabolites exchange, production of signalling molecules and genetic exchange that enhance resistance (Brooks and Flint, 2008; Burmølle et al., 2014; González-Rivas et al., 2018; Kumar and Anand, 1998). Moreover, multi-species biofilms tend to present a more complex structure (Røder et al., 2020). This is because specific matrix compounds are produced only by the community, not by the individual species grown in isolation (Guillonneau et al., 2018).

Two of the most relevant pathogens for the food industry at a European level are *Salmonella* spp. and *L. monocytogenes*. The first one is the second zoonotic agent most involved in foodborne diseases, representing 26.66% of the cases reported in 2019; and the second one is the pathogen causing the highest number of deaths with a mortality rate of 17.6% (EFSA-ECDC, 2021). Contamination by these pathogens can occur at any level of the production chain, from farms with few hygienic measures to the food industry, due to contaminated surfaces, floors, drains, and utensils, among others, and even because of poor handling by food handlers (Ehuwa et al., 2021; Mazaheri et al., 2021). Both pathogens have the ability to form monospecies or mixed species biofilms in food and on different industrial surfaces (Ripolles-Avila et al., 2018a, 2019d), although mixed biofilms formed with non-pathogenic species that belong to the resident microbiota of the food industry are most frequently found (Hascoët et al., 2019). It has been highlighted that persistence of certain pathogens such as *L. monocytogenes* is related to environmental niches shared with different species of the resident microbiota found in food factories such as Actinobacteria, *Pseudomonadaceae* or *Dipodascaceae* (Fagerlund et al., 2021). Normally, biofilm communities have different social behaviors which are broadly categorized as competitive, cooperative or neutral relations (Røder et al., 2020). In this regard, Lee et al. (2014) demonstrated that the composition and spatial organization of the species within biofilms impact the survival of the entire community when exposed to stressors. Therefore, and as discussed by Fagerlund et al. (2021), the focus of future studies could change from reductionist approaches (*i.e.* monospecies biofilms) to more complex and realistic laboratory models (*i.e.* mixed biofilms), allowing a further investigation of interactions between species residing in factory environments.

While different strategies have been developed to control biofilms such as chemical (*i.e.* biocides that inactivate microorganisms), physical (*e.g.* UV, cold plasma, among others) or biological (*e.g.* bacteriophages) approaches, there seems to be no clear direction on how to deal with the risk biofilms pose (Mazaheri et al., 2020; Ripolles-Avila et al., 2020). Therefore, the evaluation and understanding of the role played by the resident microbiota in the persistence of *L. monocytogenes* and *Salmonella* spp. is essential for the development of new strategies for their control. To this end, the present study focuses on determining the ecological relationship established in biofilms between *L. monocytogenes* and *Salmonella enterica* var. Typhimurium, and *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Bacillus safensis*, *Bacillus megaterium,* and *Candida zeylanoides*. The influence of time on the generation of these structures was also evaluated.

2. Materials and methods

2.1. Surfaces

AISI 316 stainless steel coupons (2 cm in diameter and 1 mm thick) were used as a highly employed material in the design of food contact surfaces. Prior to use, the surfaces were subjected to a cleaning and disinfection procedure, as indicated in European standard UNE-EN 13697:2015. For that, first, coupons were cleaned with a non-bactericidal detergent (ADIS Higiene, Madrid, Spain) for 1 h, afterwards exposed to 70% iso-propanol (Panreac, Castellar del Vallès, Spain) for 15 min, and air-dried in a laminar flow cabinet for 30 min. To ensure that the coupons were completely sterile, they were autoclaved for 15 min at 121 °C before microbial inoculation.

2.2. Microbial cultures used and growth conditions

Seven different microorganisms were used to develop dual-species biofilms, among which *L. monocytogenes* CECT 5672 (Spanish Collection of Type Cultures; University of Valencia, Valencia, Spain) and *S. enterica* var. Typhimurium CECT 4594 (University of Valencia, Valencia, Spain) were established as target pathogens. For the dual-species biofilms, *P. fluorescens*, *P. fragi*, *B. safensis*, *B. megaterium,* and *C. zeylanoides* were used, all isolated as part of the dominant microbiota on the surfaces in a meat industry (Hascoët et al., 2019).

The strains were obtained as freeze-dried cultures preserved at 4 °C. First, the cultures were rehydrated in 9 mL of Soy Tryptone Broth (TSB; Oxoid, Madrid, Spain) and incubated at 30 °C for 48 h. The cultures were then transferred to Tryptone Soy Agar plates (TSA; Oxoid, Madrid, Spain) and incubated at 30 °C for 24 h in the case of all the bacteria and 48 h for the yeast. From this culture, isolated colonies were transferred onto TSA plates and incubated again at 30 °C for 24 h to obtain stock cultures that were kept refrigerated at 4 °C for a maximum of one month.

2.3. Inoculum preparation, surface inoculation, and incubation conditions

A subculture was performed from the stock culture by plating it on TSA plates. The plates were incubated at 30 °C for 24 h, obtaining microbial cultures in a stationary phase. The inoculum was then prepared by transferring isolated colonies to sterile 9 mL tubes of peptone water (BioMérieux, Marcy l'Etoile, France). The microbial concentration was standardized using a densitometer (Densimat, BioMérieux, Marcy l'Etoile, France) that measures turbidity in McFarland units. Isolated colonies were transferred to obtain a turbidity of 1.5 McFarland units, equivalent to 10^8 CFU/mL. Once this suspension was obtained, 100 μL were transferred to a 9.9 mL of peptone water, thus obtaining a concentration of 10^6 CFU/mL, which was used for the biofilm formation.

Dual-species biofilms were formed by preparing a suspension of 50:50 between *L. monocytogenes* or *S.* Typhimurium as target pathogens, and the rest of the microorganisms. Once the mixed species suspension was prepared, 30 μL were inoculated in the center of each coupon, resulting in an approximate surface microbial concentration of 4 log CFU/cm^2. In parallel, a control of *L. monocytogenes* and *S.* Typhimurium in monospecies biofilm was also evaluated with the same conditions as described previously.

All the inoculated surfaces were introduced into sterile Petri dishes

and subsequently placed in a humidified chamber (saturated relative humidity >90%) using pieces of paper towels that were moistened with sterile distilled water, with the objective of promoting microbial growth and the consequent formation of biofilms (Fuster-Valls et al., 2008; Ripolles-Avila et al., 2018a, 2019a, 2019b). The surfaces were incubated at 30 °C for different periods, which were selected at 72 h, 72 + 24 h, 72 + 48 h, and 72 + 72 h. A washing protocol and renewal of nutrients was established at 72 h with the aim of eliminating non-adhered microorganisms, in addition to renewal of the culture medium to enhance the formation of the biofilm structure (Ripolles-Avila et al., 2018a). This was done by, first, washing the surfaces with 3 mL of sterile distilled water in duplicate to remove loosely attached cells and other impurities, and last, by adding 30 μL of sterile TSB to the coupons to renew the nutrients. Once this procedure was finished, the surfaces were again placed in the humid chamber and incubated for the corresponding 24, 48, or 72 h.

2.4. Quantification of the microbial load

To quantify the microbial cells present in the dual-species biofilms, cells were recovered and inoculated in different culture media. To this effect, after the incubation period (*i.e.* 72 h, 72 + 24 h, 72 + 48 h, and 72 + 72 h) the surfaces were washed with 3 mL of sterile distilled water to eliminate possible non-adhered cells, and further placed in a sterile flask containing 3 g of glass beads (2 mm in diameter) and 9 mL of sterile peptone water. The flasks were then vortexed for 90 s at a frequency of 40 Hz, with the objective of detaching the cells conforming the dual-species biofilms (Ripolles-Avila et al., 2019a).

For the cell count, the resulting suspension was decimally diluted in Tryptone Saline Solution (TSS; 1 g of tryptone [BD, Madrid, Spain] and 8.5 g of sodium chloride per liter; pH 7.0 ± 0.2). Next, 1 mL of different dilutions was inoculated onto a Petri dish, subsequently adding the corresponding culture medium, TSA for the total count and ALOA (BioMérieux, Marcy l'Etoile, France) or SS (*Salmonella-Shigella* agar; Condalab, Madrid, Spain) for the specific counts of *L. monocytogenes* and *S.* Typhimurium, respectively. The plates were then incubated at 37 °C for 24 h for the counts on the selective agar and 30 °C for 48 h for the total counts. This process was carried out for each of the mixtures and the different incubation times.

2.5. Statistical analysis

The experiments were performed in triplicate on three separate days (n = 9). CFU values for *L. monocytogenes* and *S.* Typhimurium were used to calculate by difference the CFU numbers present of the microorganism faced with the pathogen. Once the microbial counts were obtained, they were subjected to a decimal logarithmic transformation to reduce the variability in the microbiological analyses. To verify the conditions prior to performing an analysis of variance, the Shapiro-Wilk test for normality was carried out on the studied populations, and the Levene test was performed to verify the equality between variances. Once the test conditions were met, the results were evaluated by analysis of variance (ANOVA), with *a posteriori* contrast using the Tukey test. The statistical software package R v 3.6.3 was used for all the tests. A value of $P < 0.05$ was established as the level of significance.

3. Results and discussion

3.1. Monospecies biofilms of L. monocytogenes and S. Typhimurium

Microbial counts obtained for the controls (*i.e.* monospecies biofilms) over the different incubation periods is shown in Fig. 1. Regarding the counts obtained from *L. monocytogenes* in the monospecies biofilms, no significant differences ($P < 0.05$) were observed between any of the evaluated incubation periods. For that, it can be presumed that neither the washing nor time affected *L. monocytogenes* counts when producing

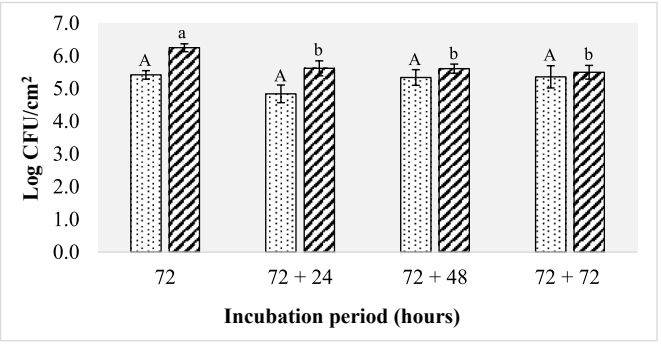


Fig. 1. Growth evolution of *Listeria monocytogenes* (□) and *Salmonella enterica* ser. Typhimurium (▨) in the monospecies biofilm during the different incubation periods. Microbial counts expressed in Log CFU/cm^2 (n = 9). Error bars indicate standard error of the mean (SEM).

biofilms under these experimental conditions. This fact is in correlation with the study conducted by Reis-Teixeira et al., (2017), which obtained no increases in sessile populations between 24 h and 192 h of incubation on stainless steel surfaces. Ripolles-Avila et al., (2018a) carried out a study for the optimization of an *L. monocytogenes* biofilm formation model, concluding that the constitution of a mature biofilm at the microscopic level was obtained at one week of incubation. Consistent with the results obtained in the present study, neither were any significant differences ($P > 0.05$) observed between the lowest incubation periods. In the case of *S.* Typhimurium, the 72-h period was the one with the greatest growth compared to the rest of the incubation periods ($P < 0.05$). This observation may be because a washing was carried out and the culture medium renewed after the first 72 h, causing the loss of non-adhered cells and other impurities. If the biofilm formation was not very compact, the washing could have caused the detachment of a large number of cells through passive desorption processes or by grazing and erosion (Krsmanovic et al., 2021), generating therefore decrease in microbial counts. This fact was also observed by Ripolles-Avila et al., (2018a) in which it was determined that reaching biofilm maturity could be compromised if *L. monocytogenes* did not have a minimum of 48 h of incubation as a period for initial attachment. Likewise, Marsh et al., (2003) observed that when the microbial concentration on a surface is low due to short incubation times, the washing and nutrient renewal process could modify the structural pattern of cell adhesion described as "honeycomb", generating a lower adhered microbial load. Nevertheless, when there was a high microbial density, these same steps did not influence the pattern of cell adhesion and as a result a higher adherent load was obtained. In the evaluated incubation periods, it was observed that *S.* Typhimuirum counts decreased non-significantly ($P > 0.05$). This could be because as incubation time passes, a degradation of nutrients occurs in biofilms due to their consumption, thus generating a decline in the number of microorganisms (Donlan, 2002; Ripolles-Avila et al., 2018a). To contrast the results obtained with their maturity level, it would be interesting to evaluate *L. monocytogenes* and *S.* Typhimuirum formed biofilms by direct epifluorescence microscopy (DEM) under the experimental conditions tested.

3.2. Impact of dominant species in the meat industry on L. monocytogenes and S. Typhimurium in dual-species biofilms

3.2.1. Pseudomonas spp. as predominant genera

Studies on the type of interaction exerted by the residential microbiota of industrial surfaces against pathogens of the food industry are nowadays highly interesting. This is because these could be used for the development of new control strategies such as biocontrol alternatives in the future (Ripolles-Avila et al., 2019c). In this regard, it has been shown that among Gram-negative microbiota the predominance of *Pseudomonas* spp. stands out (Røder et al., 2016; Stellato et al., 2016), and

especially *P. fluorescens* and *P. fragi* (Hascoët et al., 2019). A predominance of *P. fluorescens* over *L. monocytogenes* can be observed in all the experimental conditions studied in the present research (Fig. 2-A1), coinciding with findings reported by Haddad et al., (2021) regarding dominance of *P. fluorescens* over *L. monocytogenes* although without influencing pathogen counts when compared to the monospecies biofilms. According to the results of Pang and Yuk (2019), the presence of *L. monocytogenes* does not affect *P. fluorescens* cell density and it can constitute up to 90% of the population of these structures. This shows a competitive advantage under the applied conditions of *Pseudomonas* spp., which can be attributed to its growth capacity and rapid utilization of nutrients over *L. monocytogenes* in dual-species biofilms. This same dominance of *Pseudomonas* spp. was demonstrated by Heir et al. (2018), obtaining significantly lower counts of *L. monocytogenes* in the presence of *P. fluorescens* and *P. fragi*. Furthermore, the results of the confrontation between *L. monocytogenes* and *P. fragi* in dual-species biofilms showed a clear predominance of *P. fragi* over the pathogen in all conditions (Fig. 2-A.2). Sasahara and Zottola (1993) reported that a mixed culture with *P. fragi* improves the production of exopolymers and the binding and formation of colonies of *L. monocytogenes*. In the same study, it was hypothesized that *P. fragi* produced a substance that attracted *L. monocytogenes* cells, demonstrating a high consolidation of mixed biofilms. However, in the present study, no significant differences were obtained in the abundance of the pathogen in the presence of *P. fragi* among any of the times studied ($P = 0.34$; $P = 0.91$; $P = 0.82$). In contrast, Norwood and Gilmour (2001) supported the decrease in competitiveness of *L. monocytogenes* in the presence of *P. fragi* due to competition for nutrients. Furthermore, they also suggest the production of antagonist compounds together with *Staphylococcus xylosus*.

In the case of *S.* Typhimurium, the results showed that for both *P. fluorescens* and *P. fragi* the percentage of the microbial population was significantly higher than that of the pathogen itself ($P < 0.05$), presenting consequently a greater abundance (Fig. 2-B.1 and **B.2**). However, *S.* Typhimurium counts when compared with its control (*i.e.* monospecie biofilm) did not present significant differences ($P > 0.05$) with pseudomonads dual-biofilms. This could also be because *Pseudomonas* spp. presents a fast-growing pattern (Thirup et al., 2000), which could lead to a greater predominance within the structure. From the beginning, a greater predominance of both *Pseudomonas* spp. was observed. Nevertheless, at 72 h, after the washing and nutrients renewal, there was a decrease in *Pseudomonas* spp. and an increase in *S.* Typhimurium cell abundance. Similarly, Ripolles-Avila et al. (2019b) observed that *P. fragi* does not adhere with the necessary force to the surface, implying that part of the biofilm structure can be eliminated during washing, reducing population numbers. Furthermore, an increase of *S.* Typhimurium presence, mixed with *P. fluorescens* when the abundance was slightly higher, was observed 24 h after washing and renewal of the culture medium. This fact could mean that *Pseudomonas* spp. develops colonization and biofilm formation in the upper parts of the structure, something that has also been proven by other authors (Puga et al., 2018; Ripolles-Avila et al., 2019b). All this could imply that if *L. monocytogenes* or *S.* Typhimurium are found in the deepest parts of the biofilm under industrial conditions and a cleaning procedure is applied to industrial surfaces, both pathogens could be released and mobilized outside, potentially causing subsequent recontamination problems in the food industry.

Table 1 shows the derived cell counts within the formed biofilms after the different incubation periods. In general, no significant differences ($P > 0.05$) were observed between the controls (*i.e.* monospecies *L. monocytogenes* biofilms) and the *L. monocytogenes* counts obtained from the study groups (*i.e.* dual-species *P. fluorescens* and *P. fragi* biofilms). This indicates that *L. monocytogenes* presence and counts were not influenced by either *Pseudomonas* species employed in the present study. Of note is that *L. monocytogenes* cell counts increased after washing and renewal of the nutrients, although without significant differences ($P > 0.05$). In this context, a reduction of *P. fluorescens* was observed after the application of a wash and renewal of nutrients. This could be due to a greater *L. monocytogenes* adhesion to stainless steel surfaces or to an exterior placement of *P. fluorescens* within the biofilm, as observed by Puga et al. (2018). These authors pointed out that *P. fluorescens* is found in the outermost layer of the biofilm, generating an overproduction of matrix for its protection (Puga et al., 2016, 2018). By applying the wash, some *Pseudomonas* spp. cells could then be detached from the top layers, but *L. monocytogenes* could not as the pathogen would be protected in the generated structure. This is an interesting finding since it could indicate that when *Pseudomonas* spp. is present in mixed biofilms in food facilities, *L. monocytogenes* may not disappear, thus becoming an initial hypothesis for an industrial study as can be translated to two potential problems: (i) the detection of the pathogen on surfaces can be difficult with the usual sampling techniques, what could cause contaminated points to be underestimated and give therefore a false sense of safety; and (ii), after applying a regular sanitization process there could be a

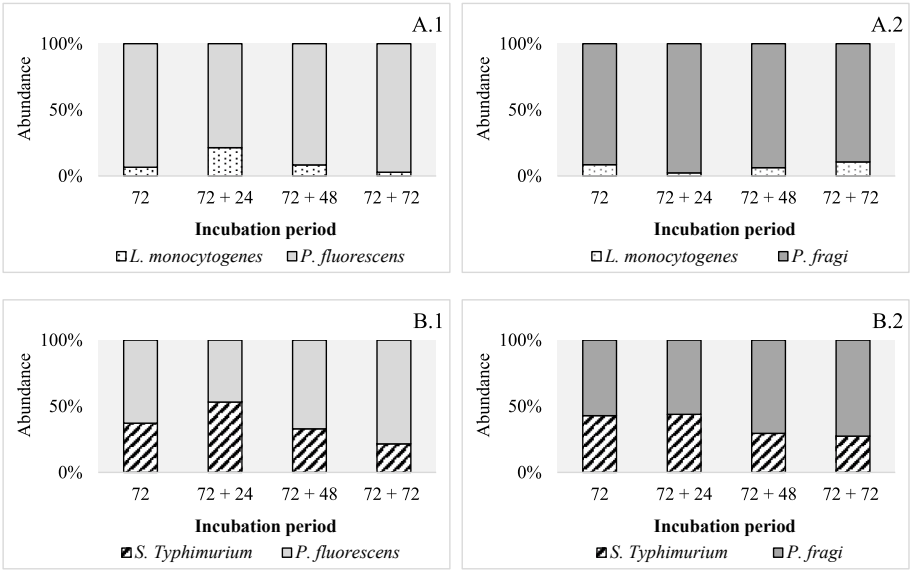


Fig. 2. Comparison of the abundance percentage (%) of *L. monocytogenes* (A) and *S.* Typhimurium (B) when forming dual-species biofilms with *P. fluorescens* (1) and *P. fragi* (2) in the 4 different incubation periods evaluated.

Table 1
Growth evolution of *L. monocytogenes* and *S.* Typhimurium in dual-species biofilms with both *Pseudomonas* species during the different incubation periods and their comparison in mono-species biofilms. Counts expressed in Log CFU/cm^2 (n = 9) ± standard error of the mean (SEM).

Pathogen's cell count in mono-species and dual-species biofilms (log CFU/cm^2)						
Incubation period (hours)	*L. monocytogenes* [a]	*L. monocytogenes* + *P. fluorescens* [a]	*L. monocytogenes* + *P. fragi* [a]	*S.* Typhimurium [b]	*S.* Typhimurium + *P. fluorescens* [b]	*S.* Typhimurium + *P. fragi* [b]
72	5.42 ± 0.13^{Aa}	5.79 ± 0.20^{Aa}	5.08 ± 0.31^{Aa}	6.25 ± 0.12^{Aa}	6.25 ± 0.11^{Aa}	6.18 ± 0.11^{Aa}
72 + 24	4.84 ± 0.27^{Aa}	5.52 ± 0.22^{Aa}	4.68 ± 0.22^{Aa}	5.62 ± 0.23^{Ba}	5.65 ± 0.16^{Ba}	5.66 ± 0.17^{Ba}
72 + 48	5.34 ± 0.24^{Aa}	5.34 ± 0.24^{Aa}	5.12 ± 0.35^{Aa}	5.61 ± 0.14^{Ba}	5.72 ± 0.13^{Ba}	5.55 ± 0.16^{Ba}
72 + 72	$5.36 \pm 0^{a}34^{Aa}$	4.99 ± 0.33^{Ba}	5.17 ± 0.27^{Aa}	5.50 ± 0.21^{Ba}	5.47 ± 0.14^{Ba}	5.72 ± 0.14^{Ba}

[A–B] Values of a column lacking a common uppercase superscript differ significantly ($P < 0.05$).
[a] Values of a row by study group (*i.e. L. monocytogenes* or *S.* Typhimurium) lacking a common lowercase superscript differ significantly ($P < 0.05$).
[a] For L. *monocytogenes* specific count ALOA medium was used.
[b] For *S.* Typhimurium specific count SS medium was used.

reduction in indicators such as total aerobic microbiota, which could facilitate the mobilization of microorganisms within the bottom layers of the biofilm, leading to an increased risk of *L. monocytogenes* growth. Therefore, anti-biofilm products would need to be developed and more sensitive methods applied that allow the pathogen to be detected quickly and efficiently, as well as to be eliminated.

Despite the observed trend, non-significant differences were found between 72 h, 72 h + 24 h, and 72 h + 48 h ($P = 0.47$; $P = 0.23$, respectively). However, in the period 72 h + 72 h, *L. monocytogenes* growth in the presence of *P. fluorescens* was significantly lower ($P < 0.05$) compared to the rest of the conditions, albeit presenting an elevated cell count. One of the possible reasons could be that *P. fluorescens* produces a bioactive substance in a late growth stage that could affect the pathogen under study. In this regard, Sharma et al. (2020) identified different compounds when combined in co-culture with *L. monocytogenes*, concluding that bioinoculants could potentially be implemented in agricultural amendments. Nevertheless, with the obtained results, this is an approach that need not be considered. Moreover, regarding the cell counts obtained from the dual-species biofilms of *L. monocytogenes* and *P. fragi*, it was observed that in all conditions *L. monocytogenes* growth was reduced by *P. fragi* presence, although no significant differences were observed in any of the incubation periods ($P = 0.41$; $P = 0.68$; $P = 0.61$; $P = 0.64$, respectively).

The same tendency was observed in the case of *S.* Typhimurium when mixed in dual-species biofilms with both *Pseudomonas* species (*i.e.* no significant differences were found between experimental groups). However, a significant reduction in *S.* Typhimurium cell numbers ($P < 0.05$) was obtained after the washing and the nutrients renewal (*i.e.* 72 h) in all the cases studied (*i.e.* in monospecies and *Pseudomonas* spp. dual-species biofilms) (Table 2). In this line, in the last incubation period (*i.e.* 72 h + 72 h), the final *S.* Typhimurium count in the biofilm formed by *P. fragi* did not present significant differences ($P > 0.05$) in comparison with the monospecies biofilms. Therefore, the *S.* Typhimurium population level was not influenced by any of the *Pseudomonas* species used, with practically the same cellular count obtained ($P > 0.05$). This fact agrees with results reported by Pang et al. (2020), in which *P. fluorescens* presence did not contribute to the development of *Salmonella* spp. biofilms. However, Pang et al. (2017) observed that the presence of *Pseudomonas aeruginosa* had an antagonistic effect against *Salmonella* spp., reducing its population level. Further studies will be required to evaluate the specific mechanisms that biofilm communities use for their social behaviors to transfer the *in vitro* results to an industrial level.

3.2.2. *Bacillus spp. as predominant genera*

Regarding the interaction with *L. monocytogenes*, the results showed a clear predominance of *B. safensis* and *B. megaterium* in all the conditions studied (Fig. 3-A.1 and **A.2**). These results coincide with those reported by Hascoët et al., (2021), although in their study a preimplantation of *Bacillus* spp. was performed, differing from the present study where the effect was evaluated in biofilms formed at the same time by direct interaction. Moreover, in the *B. safensis* case, and differently from the results obtained for *P. fluorescens*, the washing did not influence *L. monocytogenes* abundance between the 4 times studied ($P = 0.98$; $P = 0.91$, and $P = 1.00$, respectively, and compared with 72 h as the first incubation period). In terms of bacterial counts, Table 2 shows that after washing and nutrient renewal, a significant decrease ($P < 0.05$) in the *L. monocytogenes* counts was observed, although at 72 h + 48 h and 72 h + 72 h the pathogen counts increased until reaching pre-wash values ($P = 0.71$ and $P = 0.60$, respectively). Nevertheless, *L. monocytogenes* counts were significantly ($P < 0.05$) lower when confirming dual-species biofilms with *B. safensis* and *B. megaterium* in comparison with *L. monocytogenes* monospecies biofilms. In this regard, and for the case of *B. safensis*, a reduction of between 3.5 and 4 log CFU/cm^2 in the *L. monocytogenes* counts were obtained. This effect could be explained by antibacterial substance production by these type of microorganisms, including gramicidin (Yang and Yousef, 2018). In this regard, Hascoët et al., (2021) generated an extract derived from the *B. safensis* growth, demonstrating that the higher the concentration of the extract when testing it against *L. monocytogenes* the greater the delay in the pathogen's

Table 2
Growth evolution of *L. monocytogenes* and *S.* Typhimurium in dual-species biofilms with both *Bacillus* species during the different incubation periods and their comparison in mono-species biofilms. Counts expressed in Log CFU/cm^2 (n = 9) ± standard error of the mean (SEM).

Pathogen's cell count in mono-species and dual-species biofilms (log CFU/cm^2)						
Incubation period (hours)	*L. monocytogenes* [a]	*L. monocytogenes* + *B. safensis* [a]	*L. monocytogenes* + *B. megaterium* [a]	*S.* Typhimurium [b]	*S.* Typhimurium + *B. safensis* [b]	*S.* Typhimurium + *B. megaterium* [b]
72	5.42 ± 0.13^{Aa}	1.69 ± 0.38^{Ac}	3.5 ± 0.27^{Ab}	6.25 ± 0.12^{Aa}	5.57 ± 0.14^{Ab}	5.28 ± 0.19^{Ab}
72 + 24	4.84 ± 0.27^{Aa}	0.63 ± 0.17^{Bc}	3.49 ± 0.3^{Aba}	5.62 ± 0.23^{Ba}	5.63 ± 0.20^{Aa}	5.10 ± 0.23^{Aaa}
72 + 48	5.34 ± 0.24^{Aa}	1.5 ± 0.24^{Ac}	3.55 ± 0.33^{Aba}	5.61 ± 0.14^{Ba}	5.12 ± 0.16^{Aa}	5.47 ± 0.39^{Aaa}
72 + 72	5.36 ± 0.34^{Aa}	1.95 ± 0.52^{Ac}	3.5 ± 0.39^{Ab}	5.50 ± 0.21^{Ba}	4.23 ± 0.28^{Bb}	5.17 ± 0.47^{Aba}

[A–B] Values of a column lacking a common uppercase superscript differ significantly ($P < 0.05$).
[a] Values of a row by study group (*i.e. L. monocytogenes* or *S.* Typhimurium) lacking a common lowercase superscript differ significantly ($P < 0.05$).
[a] For L. *monocytogenes* specific count ALOA medium was used.
[b] For *S.* Typhimurium specific count SS medium was used.

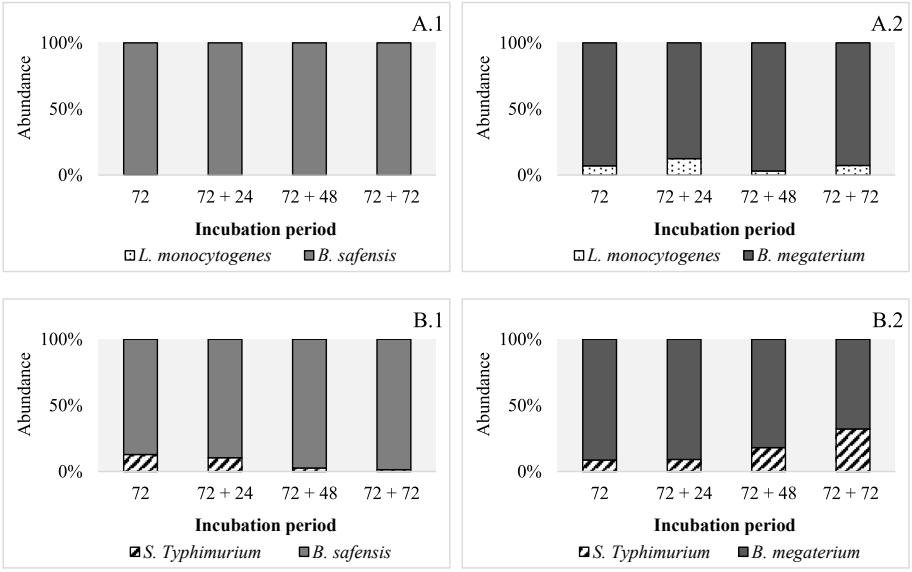


Fig. 3. Comparison of the abundance percentage (%) of *L. monocytogenes* (A) and *S.* Typhimurium (B) when forming dual-species biofilms with *B. safensis* (1) and *B. megaterium* (2) in the 4 different incubation periods evaluated.

growth, highlighting the fact that the microorganism can be producing an antibacterial substance. Moreover, Hascoët et al., (2021) demonstrated that *L. monocytogenes* counts decreased significantly in the presence of preformed *B. safensis* biofilms, while these preformed biofilms were not affected and stable over time. This fact was again corroborated by Cervantes-Huaman (2019) who demonstrated that *L. monocytogenes* did not adhere adequately to the surface and produce a robust biofilm when *B. safensis* was present on the surface as a preformed biofilm. All these findings in conjunction with the ones reported in the present study suggest that *B. safensis* could be an interesting species for *L. monocytogenes* replacement on industrial surfaces and a potential species for the biocontrol of this pathogen. When L. *monocytogenes* counts in monospecies biofilms were compared with the ones obtained in dual-species biofilms with *B. megaterium*, a significant decrease in the counts of the pathogen of between 1.5 and 2 log CFU/cm^2 were obtained. However, the global effect was not as noticeable as with *B. safensis*. The obtained results were in concordance with Barbosa et al., (2005), who reported similar antimicrobial activities by *B. megaterium* against *L. monocytogenes*.

Sharma et al., (2020) did not find significant differences between *B. megaterium* populations and *L. monocytogenes* presence or absence, implying that the pathogen does not influence *B. megaterium* growth. In this last study, an extraction was carried out and different bioactive compounds were identified, which could also explain the anti-*Listeria* effect and the results obtained in this study.

In the case of *S.* Typhimurium, and in terms of abundance, the predominance of *B. safensis* and *B. megaterium* was way superior to that presented by the tested pathogen (Fig. 3-B.1 and **B.2**), coinciding with the results obtained for *L. monocytogenes*. This higher abundance could be explained by the report that *Bacillus* spp. is a bacterial genera with a strong expansion capacity when conforming biofilms, even able to double in size in just four days (Gingichashvili et al., 2017). As the incubation periods elapsed, the tendency of *B. safensis* and *B. megaterium* and their interaction with *S.* Typhimurium was the opposite. The predisposition of *B. safensis* was to increase the percentage of its population and therefore to decrease the abundance of the pathogen in the dual-species biofilms, while in the case of *B. megaterium* its abundance was reduced to be displaced by *S.* Typhimurium, which increased its population. Despite this, both species of *Bacillus* spp. dominated over *S.* Typhimurium.

In terms of cell counts, when conforming dual-species biofilms with *S.* Typhimurium both *B. safensis* and *B. megaterium* significantly ($P < 0.05$) reduced the pathogen counts in the 72 h and 72 h + 72 h incubation periods (Table 2), although under no test conditions was the observed reduction as high as for *L. monocytogenes*. This result could be explained by this species of *Bacillus* having a faster growth than *S.* Typhimurium, thus producing a conflict over nutrients and hindering the development of the pathogen. Moreover, when *Bacillus* spp. reaches the stationary phase (*i.e.* a phase that could be happening at 72 h or at 72 h + 72 h) phenomena such as nutrient competition, the decrease in essential factors for respiration, or even variation at a more acidic pH can occur (Castañeda-Alvarez and Consuelo-Sánchez, 2016). The spatial distribution of the microbial species within the biofilm could also affect the ecological relation and subsequent treatment choice for their elimination (Rodríguez-Melcón et al., 2021). There could be a social conflict between the bacteria that are located on the periphery and those that are inside the biofilm, since those that are outside consume most of the resources, notoriously limiting and depriving the nutrients that reach the interior.

3.2.3. *Candida spp. as predominant genera*

Within the fungal diversity established in the different food industries, there is a predominance of the genus *Candida* spp. (Gounadaki et al., 2008). In the study carried out by Hascoët et al. (2019) different species of *Candida* spp. were isolated, among which *C. krusei*, *C. ciferry*, *C. zeylanoides* were reported, the latter predominating at a percentage of 61%. In the results obtained in the present study, a clear predominance of *C. zeylanoides* with respect to *L. monocytogenes* was observed at all incubation times studied (Fig. 4-A). Specifically, when comparing *L. monocytogenes* cell counts on the monospecies biofilms and when conforming dual-species biofilms with *C. zeylanoides* (Table 3), differences of up to more than 1 log ($P < 0.05$) were observed, indicating that *C. zeylanoides* slows *L. monocytogenes* growth. No studies have been done to understand the relation with *C. zeylanoides* and *L. monocytogenes* in dual-species biofilms. However, notable is the study carried out by Agustín and Brugnoni (2018), in which dual-species biofilms with *L. monocytogenes* and *Listeria innocua* with *Candida tropicalis*, *Candida kefyr,* and *C. krusei* were generated. They concluded that *L. monocytogenes* increases its presence with *C. tropicalis* and *L. innocua* in the presence of *C. tropicalis* and *C. krusei*. However, *L. innocua* decreases its presence with *C. kefyr*. Between these results and those obtained in the present study, antagonistic and synergistic interactions between

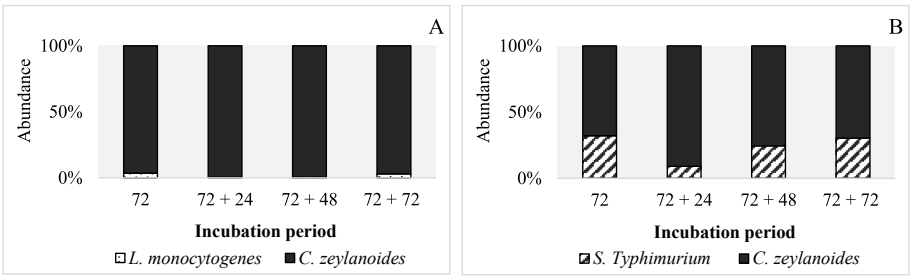


Fig. 4. Comparison of the abundance percentage (%) of *L. monocytogenes* (A) and *S.* Typhimurium (B) when forming dual-species biofilms with *C. zeylanoides* in the 4 different incubation periods evaluated.

Table 3
Growth evolution of *L. monocytogenes* and *S.* Typhimurium in dual-species biofilms with *C. zeylanoides* during the different incubation periods and their comparison in mono-species biofilms. Counts expressed in Log CFU/cm^2 (n = 9) ± standard error of the mean (SEM).

	Pathogen's cell count in mono-species and dual-species biofilms (log CFU/cm^2)			
Incubation period (hours)	*L. monocytogenes* [a]	*L. monocytogenes* + *C. zeylanoides* [a]	*S.* Typhimurium [b]	*S.* Typhimurium + *C. zeylanoides* [b]
72	5.42 ± 0.13^{Aa}	4.01 ± 0.33^{Ab}	6.25 ± 0.12^{Aa}	5.64 ± 0.23^{Ab}
72 + 24	4.84 ± 0.27^{Aa}	2.97 ± 0.35^{Bb}	5.62 ± 0.23^{Ba}	5.37 ± 0.16^{Aa}
72 + 48	5.34 ± 0.24^{Aa}	3.69 ± 0.34^{Ab}	5.61 ± 0.14^{Ba}	5.76 ± 0.32^{Aa}
72 + 72	5.36 ± 0.34^{Aa}	2.82 ± 0.56^{Bb}	5.50 ± 0.21^{Ba}	5.53 ± 0.34^{Aa}

[A–B] Values of a column lacking a common uppercase superscript differ significantly ($P < 0.05$).
[a] Values of a row by study group (*i.e. L. monocytogenes* or *S.* Typhimurium) lacking a common lowercase superscript differ significantly ($P < 0.05$).
[a] For L. *monocytogenes* specific count ALOA medium was used.
[b] For *S.* Typhimurium specific count SS medium was used.

species of the same genus are revealed. Therefore, more studies are needed to understand *L. monocytogenes* behavior. The results obtained from the interaction between *C. zeylanoides* and the pathogen indicate that this resident yeast could be a good option to be used as a potential biocontrol species in food industries in conjunction with other inhibitory species of the pathogen. The search for a complementary and competitive microbiota for the replacement of pathogens could be an alternative strategy for the microbial control of industrial surfaces in the future (Hascoët et al., 2019; Ripolles-Avila et al., 2019c). However, to this end there is a need to study yeasts in greater depth, and specifically how they interact with other microbial species, since these microorganisms are the most abundant at the level of isolates and so it is essential to know their interactions.

Regarding *S.* Typhimurium abundance, at the beginning of the incubation period (*i.e.* at 72 h) there was a higher percentage of *C. zeylanoides,* although the difference became much more evident at the incubation period of 72 h + 24 h, when the population count of *C. zeylanoides* reached more than 90%, while *S.* Typhimurium abundance fell below 10% (Fig. 4-B). As the incubation period evolved, the opposite effect occurred, with the population of *S.* Typhimurium beginning to increase, while that of *C. zeylanoides* decreased to the point where, in the last period (*i.e.* 72 h + 72 h), the *S.* Typhimurium population percentage reached that obtained at 72 h. This reduction in *C. zeylanoides* could be explained by the production by *S.* Typhimurium of determinate substances such as B complex vitamins (Fang et al., 2017). In this regard, it has been indicated that certain B-complex vitamins such as Vitamin B2 (*i.e.*, riboflavin), B3 (*i.e.* pantothenic acid), and B9 (*i.e.* folic acid) have a partial antifungal effect against *Candida albicans* (Meir and Osherov, 2018).

Moreover, the results in terms of cell counts are presented in Table 3. As can be observed, the number of *S.* Typhimurium cells when conforming dual-species biofilms with *C. zeylanoides* in the first incubation period (*i.e.* 72 h) was significantly lower ($P < 0.05$) than that obtained in the monospecies biofilm, with a difference in the population counts of approximately 0.60 log CFU/cm^2. As the incubation periods elapsed, *S.* Typhimurium counts in the dual-species biofilms resembled those obtained in the monospecies biofilms, without presenting significant differences ($P > 0.05$). Viljoen (2001) stated that certain yeasts can stimulate or inhibit bacterial growth, although this depends on the degree of interrelation between them, so yeasts must be taken into consideration when considering the residential microbiota and the ecological relation they can establish with certain pathogens.

4. Conclusions

Different ecological relationships were established between the two pathogens and the dominant microbiota evaluated, demonstrating no influence on their growth or an inhibition of it depending on the microorganism. *P. fluorescens* and *P. fragi* did not enhance or inhibit the growth of *L. monocytogenes* or *S. enterica*. However, both pathogens remained in the mixed biofilm, protected by the two species. *B. safensis*, *B. megaterium,* and *C. zeylanoides* inhibit the growth of *L. monocytogenes* to different degrees when forming dual-species biofilms, achieving a final reduction in the number of adhered cells. However, in the case of *S. enterica*, *B. safensis* was the only microorganism that exerted this outcompeting effect. The results have a potential technological use in the future to control foodborne pathogens and reduce the use of detergents and disinfectants. However, more studies must be carried out to corroborate the viability of this approach in terms of application, determine possible microorganisms with the same or greater inhibitory effect, and specify the best combination of microorganisms to control certain pathogens considered high risk.

Funding sources

This study was supported by Research Project grants RTI2018-098267-R-C32 from the Ministerio de Ciencia, Innovación y Universidades.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Dolors Busquets profusely for her technical assistance. The authors alone are responsible for the content and writing of the paper. The authors acknowledge Ms. Sarah Davies for the English grammar review.

References

Agustín, M. del R., Brugnoni, L., 2018. Multispecies biofilms between *Listeria monocytogenes* and *Listeria innocua* with resident microbiota isolated from apple juice processing equipment. J. Food Saf. 38, e12499.

Álvarez-Ordóñez, A., Briandet, R., 2016. Editorial: biofilms from a food microbiology perspective: structures, functions, and control strategies. Front. Microbiol. 7, 1–3. https://doi.org/10.3389/fmicb.2016.01938.

Barbosa, T.M., Serra, C.R., La Ragione, R.M., Woodward, M.J., Henriques, A.O., 2005. Screening for *Bacillus* isolates in the broiler gastrointestinal tract. Appl. Environ. Microbiol. 71, 968–978. https://doi.org/10.1128/AEM.71.2.968-978.2005.

Bridier, A., Briandet, R., Thomas, V., Dubois-Brissonnet, F., 2011. Resistance of bacterial biofilms to disinfectants: a review. Biofouling 27, 1017–1032. https://doi.org/10.1080/08927014.2011.626899.

Brightwell, G., Boerema, J., Mills, J., Mowat, E., Pulford, D., 2006. Identifying the bacterial community on the surface of Intralox™ belting in a meat boning room by culture-dependent and culture-independent 16S rDNA sequence analysis. Int. J. Food Microbiol. 109, 47–53. https://doi.org/10.1016/j.ijfoodmicro.2006.01.008.

Brooks, J.D., Flint, S.H., 2008. Biofilms in the food industry: problems and potential solutions. Int. J. Food Sci. Technol. 43, 2163–2176. https://doi.org/10.1111/j.1365-2621.2008.01839.x.

Burmølle, M., Ren, D., Bjarnsholt, T., Sørensen, S.J., 2014. Interactions in multispecies biofilms: do they actually matter? Trends Microbiol 22, 84–91. https://doi.org/10.1016/j.tim.2013.12.004.

Capita, R., Riesco-Peláez, F., Alonso-Hernando, A., Alonso-Calleja, C., 2014. Exposure of *Escherichia coli* ATCC 12806 to sublethal concentrations of food-grade biocides influences its ability to form biofilm, resistance to antimicrobials, and ultrastructure. Appl. Environ. Microbiol. 80, 1268–1280. https://doi.org/10.1128/AEM.02283-13.

Castañeda-Alvarez, E., Consuelo-Sánchez, L., 2016. Evaluation of growth of four species of the genus *Bacillus* sp., the first step to understand their biocontrol effect on *Fusarium* sp. Nova 13, 53–65.

Cervantes-Huaman, B.R.H, 2019. Master thesis Desarrollo de biofilms monoespecie patogénicos sobre superficies de acero inoxidable y enfrentamiento contra un microorganismo de interés para su potencial biocontrol: Estudio cuantitativo y composicional. Universitat Autònoma de Barcelona.

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. Emerg. Infect. Dis. 8, 881–890. https://doi.org/10.3201/eid0809.020063.

EFSA-ECDC, 2021. The European union one health 2019 Zoonoses report. EFSA J 19, 1–286. https://doi.org/10.2903/j.efsa.2021.6406.

Ehuwa, O., Jaiswal, A.K., Jaiswal, S., 2021. *Salmonella*, food safety and food handling practices. Foods 10, 1–16. https://doi.org/10.3390/foods10050907.

Fagerlund, A., Langsrud, S., Møretrø, T., 2021. Microbial diversity and ecology of biofilms in food industry environments associated with *Listeria monocytogenes* persistence. Curr. Opin. Food Sci. 37, 171–178. https://doi.org/10.1016/j.cofs.2020.10.015.

Fagerlund, A., Langsrud, S., Møretrø, T., 2021. Microbial diversity and ecology of biofilms in food industry environments associated with *Listeria monocytogenes* persistence. Curr. Opin. Food Sci. 37, 171–178. https://doi.org/10.1016/j.cofs.2020.10.015.

Fagerlund, A., Moretro, T., Heir, E., Briandet, R., Langsrud, S., Møretrø, T., Heir, E., Briandet, R., Langsruda, S., 2017. Cleaning and disinfection of biofilms composed of *Listeria monocytogenes*. Appl. Environ. Microbiol. 83, 1–21.

Fang, H., Kang, J., Zhang, D., 2017. Microbial production of vitamin B12: a review and future perspectives. Microb. Cell Factories 16, 1–14. https://doi.org/10.1186/s12934-017-0631-y.

Flemming, H., Wingender, J., 2010. The biofilm matrix. Nat. Rev. Microbiol. 8, 623–633. https://doi.org/10.1038/nrmicro2415.

Fuster-Valls, N., Hernández-Herrero, M., Marín-de-Mateo, M., Rodríguez-Jerez, J.J., 2008. Effect of different environmental conditions on the bacteria survival on stainless steel surfaces. Food Control 19, 308–314. https://doi.org/10.1016/j.foodcont.2007.04.013.

Gingichashvili, S., Duanis-Assaf, D., Shemesh, M., Featherstone, J.D.B., Feuerstein, O., Steinberg, D., 2017. *Bacillus subtilis* biofilm development - a computerized study of morphology and kinetics. Front. Microbiol. 8, 1–9. https://doi.org/10.3389/fmicb.2017.02072.

González-Rivas, F., Ripolles-Avila, C., Fontecha-Umaña, F., Ríos-Castillo, A.G., Rodríguez-Jerez, J.J., 2018. Biofilms in the spotlight: detection, quantification, and removal methods. Compr. Rev. Food Sci. Food Saf. 17, 1261–1276. https://doi.org/10.1111/1541-4337.12378.

Gounadaki, A.S., Skandamis, P.N., Drosinos, E.H., Nychas, G.J.E., 2008. Microbial ecology of food contact surfaces and products of small-scale facilities producing traditional sausages. Food Microbiol 25, 313–323. https://doi.org/10.1016/j.fm.2007.10.001.

Guillonneau, R., Baraquet, C., Bazire, A., Molmeret, M., 2018. Multispecies biofilm development of marine bacteria implies complex relationships through competition and synergy and modification of matrix components. Front. Microbiol. 9, 1–15. https://doi.org/10.3389/fmicb.2018.01960.

Haddad, S., Elliot, M., Savard, T., Deschênes, L., Smith, T., Ells, T., 2021. Variations in biofilms harbouring *Listeria monocytogenes* in dual and triplex cultures with *Pseudomonas fluorescens* and *Lactobacillus plantarum* produced under a model system of simulated meat processing conditions, and their resistance to benzalkonium chloride. Food Control 123, 107720. https://doi.org/10.1016/j.foodcont.2020.107720.

Hascoët, A.-S., Ripolles-Avila, C., Guerrero-Navarro, A.E., Rodríguez-Jerez, J.J., 2019. Microbial ecology evaluation of an iberian pig processing plant through implementing sch sensors and the influence of the resident microbiota on *Listeria monocytogenes*. Appl. Sci. 9, 1–14. https://doi.org/10.3390/app9214611.

Hascoët, A.S., Ripolles-Avila, C., Cervantes-Huamán, B.R.H., Rodríguez-Jerez, J.J., 2021. *In vitro* preformed biofilms of *Bacillus safensis* inhibit the adhesion and subsequent development of *Listeria monocytogenes* on stainless-steel surfaces. Biomolecules 11, 1–16. https://doi.org/10.3390/biom11030475.

Heir, E., Møretrø, T., Simensen, A., Langsrud, S., 2018. *Listeria monocytogenes* strains show large variations in competitive growth in mixed culture biofilms and suspensions with bacteria from food processing environments. Int. J. Food Microbiol. 275, 46–55. https://doi.org/10.1016/j.ijfoodmicro.2018.03.026.

Krsmanovic, M., Biswas, D., Ali, H., Kumar, A., Ghosh, R., Dickerson, A., 2021. Hydrodynamics and surface properties influence biofilm proliferation. Adv. Colloid Interface Sci. 288, 102336. https://doi.org/10.1016/j.cis.2020.102336.

Kumar, C.G., Anand, S.K., 1998. Significance of microbial biofilms in food industry: a review. Int. J. Food Microbiol. 42, 9–27. https://doi.org/10.1016/S0168-1605(98)00060-9.

Lee, K.W.K., Periasamy, S., Mukherjee, M., Xie, C., Kjelleberg, S., Rice, S.A., 2014. Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. ISME J 8, 894–907. https://doi.org/10.1038/ismej.2013.194.

Marsh, E.J., Luo, H., Wang, H., 2003. A three-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities. FEMS Microbiol. Lett. 228, 203–210. https://doi.org/10.1016/S0378-1097(03)00752-3.

Mazaheri, T., Cervantes-Huamán, B.R.H., Bermúdez-Capdevila, M., Ripolles-Avila, C., Rodríguez-Jerez, J.J., 2021. *Listeria monocytogenes* biofilms in the food industry: is the current hygiene program sufficient to combat the persistence of the pathogen? Microorganisms 9, 1–19. https://doi.org/10.3390/microorganisms9010181.

Mazaheri, T., Ripolles-Avila, C., Hascoët, A.-S., Rodríguez-Jerez, J.J., 2020. Effect of an enzymatic treatment on the removal of mature *Listeria monocytogenes* biofilms: a quantitative and qualitative study. Food Control 114, 107266. https://doi.org/10.1016/j.foodcont.2020.107266.

Meir, Z., Osherov, N., 2018. Vitamin biosynthesis as an antifungal target. J. Fungi 4, 1–14. https://doi.org/10.3390/jof4020072.

Molina-González, D., Alonso-Calleja, C., Alonso-Hernando, A., Capita, R., 2014. Effect of sub-lethal concentrations of biocides on the susceptibility to antibiotics of multi-drug resistant *Salmonella enterica* strains. Food Control 40, 329–334. https://doi.org/10.1016/j.foodcont.2013.11.046.

Møretrø, T., Langsrud, S., 2017. Residential bacteria on surfaces in the food industry and their implications for food safety and quality. Compr. Rev. Food Sci. Food Saf. 16, 1022–1041. https://doi.org/10.1111/1541-4337.12283.

Norwood, D.E., Gilmour, A., 2001. The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. Lett. Appl. Microbiol. 33, 320–324. https://doi.org/10.1046/j.1472-765X.2001.01004.x.

Pang, X., Chen, L., Yuk, H.G., 2020. Stress response and survival of *Salmonella* Enteritidis in single and dual species biofilms with *Pseudomonas fluorescens* following repeated exposure to quaternary ammonium compounds. Int. J. Food Microbiol. 325, 108643. https://doi.org/10.1016/j.ijfoodmicro.2020.108643.

Pang, X., Yuk, H.G., 2019. Effects of the colonization sequence of *Listeria monocytogenes* and *Pseudomonas fluorescens* on survival of biofilm cells under food-related stresses and transfer to salmon. Food Microbiol 82, 142–150. https://doi.org/10.1016/j.fm.2019.02.002.

Pang, X.Y., Yang, Y.S., Yuk, H.G., 2017. Biofilm formation and disinfectant resistance of *Salmonella* sp. in mono- and dual-species with Pseudomonas aeruginosa. J. Appl. Microbiol. 123, 651–660. https://doi.org/10.1111/jam.13521.

Puga, C.H., Dahdouh, E., SanJose, C., Orgaz, B., 2018. *Listeria monocytogenes* colonizes *Pseudomonas fluorescens* biofilms and induces matrix over-production. Front. Microbiol. 9, 1–12. https://doi.org/10.3389/fmicb.2018.01706.

Puga, C.H., Orgaz, B., SanJose, C., 2016. *Listeria monocytogenes* impact on mature or old *Pseudomonas fluorescens* biofilms during growth at 4 and 20°C. Front. Microbiol. 7, 1–9. https://doi.org/10.3389/fmicb.2016.00134.

Reis-Teixeira, F.B. dos, Alves, V.F., de Martinis, E.C.P., 2017. Growth, viability and architecture of biofilms of *Listeria monocytogenes* formed on abiotic surfaces. Braz. J. Microbiol. 48, 587–591. https://doi.org/10.1016/j.bjm.2017.01.004.

Ripolles-Avila, C., Cervantes-Huaman, B.H., Hascoët, A.-S., Yuste, J., Rodríguez-Jerez, J. J., 2019a. Quantification of mature *Listeria monocytogenes* biofilm cells formed by an *in vitro* model: a comparison of different methods. Int. J. Food Microbiol. 289, 209–214. https://doi.org/10.1016/j.ijfoodmicro.2018.10.020.

Ripolles-Avila, C., García-Hernández, N., Cervantes-Huamán, B.H., Mazaheri, T., Rodríguez-Jerez, J.J., 2019b. Quantitative and compositional study of monospecies biofilms of spoilage microorganisms in the meat industry and their interaction in the development of multispecies biofilms. Microorganisms 7, 1–14. https://doi.org/10.3390/microorganisms7120655.

Ripolles-Avila, C., Hascoët, A.-S., Guerrero-Navarro, A.E., Rodríguez-Jerez, J.J., 2018a. Establishment of incubation conditions to optimize the *in vitro* formation of mature *Listeria monocytogenes* biofilms on food-contact surfaces. Food Control 92, 240–248. https://doi.org/10.1016/j.foodcont.2018.04.054.

Ripolles-Avila, C., Hascoët, A.-S., Martínez-Suárez, J.V., Capita, R., Rodríguez-Jerez, J.J., 2019c. Evaluation of the microbiological contamination of food processing environments through implementing surface sensors in an iberian pork processing plant: an approach towards the control of *Listeria monocytogenes*. Food Control 99, 40–47. https://doi.org/10.1016/j.foodcont.2018.12.013.

Ripolles-Avila, C., Ramos-Rubio, M., Hascoët, A.S., Castillo, M., Rodríguez-Jerez, J.J., 2020. New approach for the removal of mature biofilms formed by wild strains of *Listeria monocytogenes* isolated from food contact surfaces in an Iberian pig processing plant. Int. J. Food Microbiol. 323, 108595. https://doi.org/10.1016/j.ijfoodmicro.2020.108595.

Ripolles-Avila, C., Ríos-Castillo, A.G., Fontecha-Umaña, F., Rodríguez-Jerez, J.J., 2019d. Removal of *Salmonella enterica* serovar Typhimurium and *Cronobacter sakazakii* biofilms from food contact surfaces through enzymatic catalysis. J. Food Saf. 40, e12755 https://doi.org/10.1111/jfs.12755.

Ripolles-Avila, C., Ríos-Castillo, A.G., Rodríguez-Jerez, J., 2018b. Development of a peroxide biodetector for a direct detection of biofilms produced by catalase-positive bacteria on food-contact surfaces. CyTA - J. Food 16, 506–515. https://doi.org/10.1080/19476337.2017.1418434.

Røder, H.L., Olsen, N.M.C., Whiteley, M., Burmølle, M., 2020. Unravelling interspecies interactions across heterogeneities in complex biofilm communities. Environ. Microbiol. 22, 5–16. https://doi.org/10.1111/1462-2920.14834.

Røder, H.L., Sørensen, S.J., Burmølle, M., 2016. Studying bacterial multispecies biofilms: where to start? Trends Microbiol 24, 503–513. https://doi.org/10.1016/j.tim.2016.02.019.

Rodríguez-Melcón, C., Alonso-Hernando, A., Riesco-Peláez, F., García-Fernández, C., Alonso-Calleja, C., Capita, R., 2021. Biovolume and spatial distribution of foodborne Gram-negative and Gram-positive pathogenic bacteria in mono- and dual-species biofilms. Food Microbiol 94. https://doi.org/10.1016/j.fm.2020.103616.

Rodríguez-Melcón, C., Capita, R., Rodríguez-Jerez, J.J., Martínez-Suárez, J.V., Alonso-Calleja, C., 2019. Effect of low doses of disinfectants on the biofilm-forming ability of *Listeria monocytogenes*. Foodb. Pathog. Dis. 16, 262–268. https://doi.org/10.1089/fpd.2018.2472.

Sasahara, K.C., Zottola, E.A., 1993. Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. J. Food Protect. 56, 1022–1028. https://doi.org/10.4315/0362-028X-56.12.1022.

Sharma, R., Gal, L., Garmyn, D., Bisaria, V.S., Sharma, S., Piveteau, P., 2020. Evidence of biocontrol activity of bioinoculants against a human pathogen, *Listeria monocytogenes*. Front. Microbiol. 11, 1–13. https://doi.org/10.3389/fmicb.2020.00350.

Speranza, B., Monacis, N., Sinigaglia, M., Corbo, M.R., 2017. Approaches to removal and killing of *Salmonella* spp. biofilms. J. Food Process. Preserv. 41, 1–9. https://doi.org/10.1111/jfpp.12758.

Stellato, G., La Storia, A., De Filippis, F., Borriello, G., Villani, F., Ercolini, D., 2016. Overlap of spoilage-associated microbiota between meat and the meat processing environment in small-scale and large-scale retail distributions. Appl. Environ. Microbiol. 82, 4045–4054. https://doi.org/10.1128/AEM.00793-16.

Thirup, L., Ekelund, F., Johnsen, K., Jacobsen, C.S., 2000. Population dynamics of the fast-growing sub-populations of *Pseudomonas* and total bacteria, and their protozoan grazers, revealed by fenpropimorph treatment. Soil Biol. Biochem. 32, 1615–1623. https://doi.org/10.1016/S0038-0717(00)00075-4.

Viljoen, B.C., 2001. The interaction between yeasts and bacteria in dairy environments. Int. J. Food Microbiol. 69, 37–44. https://doi.org/10.1016/S0168-1605(01)00570-0.

Yang, X., Yousef, A.E., 2018. Antimicrobial peptides produced by *Brevibacillus* spp.: structure, classification and bioactivity: a mini review. World J. Microbiol. Biotechnol. 34, 1–10. https://doi.org/10.1007/s11274-018-2437-4.