International Journal of Food Microbiology 303 (2019) 32–41

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Kinetics of biofilm formation by pathogenic and spoilage microorganisms under conditions that mimic the poultry, meat, and egg processing industries

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ARTICLE INFO

Keywords:
Multispecies biofilms
Food contact surfaces
Food industry

ABSTRACT

Pathogens and spoilage microorganisms can develop multispecies biofilms on food contact surfaces; however, few studies have been focused on evaluated mixed biofilms of these microorganisms. Therefore this study investigated the biofilm development by pathogenic (*Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* Enteritidis and Typhimurium serotypes) and spoilage (*Bacillus cereus* and *Pseudomonas aeruginosa*) microorganisms onto stainless-steel (SS) and polypropylene B (PP) coupons; under conditions that mimic the dairy, meat, and egg processing industry. Biofilms were developed in TSB with 10% chicken egg yolk (TSB + EY), TSB with 10% meat extract (TSB + ME) and whole milk (WM) onto SS and PP. Each tube was inoculated with 25 μL of each bacteria and then incubated at 9 or 25 °C, with enumeration at 1, 48, 120, 180 and 240 h. Biofilms were visualized by epifluorescence and scanning electron microscopy (SEM). Biofilm development occurred at different phases, depending on the incubation conditions. In the reversible adhesion, the cell density of each bacteria was between 1.43 and 6.08 Log_{10} CFU/cm^2 ($p < 0.05$). Moreover, significant reductions in bacteria appeared at 9 °C between 1 and 48 h of incubation. Additionally, the constant multiplication of bacteria in the biofilm occurred at 25 °C between 48 and 180 h of incubation, with increments of 2.08 Log_{10} CFU/cm^2 to *S.* Typhimurium. Population establishment was observed between 48 and 180 h and 180–240 h incubation, depending on the environmental conditions (25 and 9 °C, respectively). For example, in TSB + ME at 25 °C, *S.* Typhimurium, *P aeruginosa*, and *L. monocytogenes* showed no statistical differences in the amounts between 48 and 180 h incubation. The dispersion phase was identified for *L. monocytogenes* and *B. cereus* at 25 °C. Epifluorescence microscopy and SEM allowed visualizing the bacteria and extracellular polymeric substances at the different biofilm stages. In conclusion, pathogens and spoilage microorganisms developed monospecies with higher cellular densities than multiespecies biofilms. In multispecies biofilms, the time to reach each biofilm phase varied is depending on environmental factors. Cell count decrements of 1.12–2.44 Log_{10} CFU/cm^2 occurred at 48 and 240 h and were most notable in the biofilms developed at 9 °C. Additionally, cell density reached by each microorganism was different, *P. aeruginosa* and *Salmonella* were the dominant microorganisms in the biofilms while *B. cereus* showed the lower densities until undetectable levels.

1. Introduction

Biofilms are defined as a community of microorganisms (of the same or different species) enclosed in an of self-produced extracellular polymeric substances (EPS) matrix that is able to adhere strongly to both living and abiotic surfaces (Steenackers et al., 2012); these communities are the main mode of growth of bacteria in the environment (Giaouris et al., 2015). Biofilm formation has been recognized as a strategy used by microorganisms to survive in hostile environments, such as on inert surfaces exposed to hostile conditions (UV light, desiccation, heat, cold, shear forces). Additionally, organisms within a biofilm are far more resistant to antimicrobial agents than planktonic

Abbreviations: CFDA, 5(6)-carboxyfluorescein diacetate; EPS, extracellular polymeric substances; EY, egg yolk; ME, meat extract; PP, polypropylene B; SS, stainless-steel; WM, whole milk

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https://doi.org/10.1016/j.ijfoodmicro.2019.04.012
Received 12 November 2018; Received in revised form 29 April 2019; Accepted 30 April 2019
Available online 11 May 2019
0168-1605/

cells (Almeida et al., 2011; Mah and O'Toole, 2001). Biofilm formation is a dynamic process with different phases: i) reversible attachment, ii) desorption, iii) irreversible attachment, iv) microcolonies development, v) maturation and vi) dispersion (Srey et al., 2013; Stoodley et al., 2002). This process is affected by several factors, such as temperature, pH, nutrients content, salinity, contact surface properties, and microorganism proprieties (Donlan, 2002; Xu et al., 2010).

In the food industry the pathogenic and spoilage microorganisms develop biofilms on food contact surfaces (Giaouris et al., 2013b); that entails equipment contamination, biofouling in drinking water systems, and post-process contamination, contributing to food spoilage and foodborne diseases (Chmielewski and Frank, 2003; Zhao et al., 2017). In line with this, it has been estimated that every year around 600 million people fall ill after eating contaminated food in the world; including almost 350 million caused by pathogenic bacteria; this constitute a serious health risk both in developing countries and in advanced ones such as the United States (WHO, 2017). In this country t has been estimated that *Salmonella* causes 1.2 million of foodborne diseases every year (CDC, 2019); the serotype Typhimurium has been associated to the meat and dairy industries whereas the serotype Enteritidis to the poultry industry (Lamas et al., 2018; Yang et al., 2017). Other important biofilm-former microorganisms commonly implicated in foodborne diseases are *Listeria monocytogenes* (Ripolles-Avila et al., 2019), *Escherichia coli* (Corzo-Ariyama et al., 2019), and *Bacillus cereus* (Park et al., 2019). Moreover, in the last decades, the dairy, egg processing, and meat industries have focused extensively on the management of food spoilage caused by biofilm-forming microorganisms, such as *Clostridium*, *Brochothrix thermosphacta*, Enterobacteriaceae, lactic acid bacteria, *Pseudomonas* spp., and *Bacillus* spp. (Majed et al., 2016; Meliani and Bensoltane, 2015; Motarjemi and Lelieveld, 2014). Due to according to the Food and Agricultural Organization of the United Nations (FAO, 2011), at least 25% of global food production is deteriorated by microbial action (bacteria, yeasts, and molds).

Previous researches showed that *L. monocytogenes*, *P. aeruginosa*, *B. cereus*, *Salmonella* spp., and *E. coli* can develop dual- or tri-species biofilms with Gram-positive and Gram-negative species (Almeida et al., 2011; Bridier et al., 2012; Giaouris et al., 2013a; Iñiguez-Moreno et al., 2018; Kostaki et al., 2012). However, few have reported the biofilm formation by pathogenic and spoilage microorganisms (Giaouris et al., 2013b; Guillier et al., 2008; Mellefont et al., 2008; van der Veen and Abee, 2011) and most have focused on co-culture conditions, without considering that biofilms in the food industry are formed by multiple bacteria (Kostaki et al., 2012; Nozhevnikova et al., 2015). To our knowledge, no prior research has reported biofilm development by five microorganisms under conditions that mimic the food industry. Hence, this aim of this study was investigated the mono- and multispecies biofilm development by pathogenic (*B. cereus*, *E. coli*, *L. monocytogenes*, and *S. enterica*) and spoilage (*B. cereus* and *P. aeruginosa*) microorganisms onto stainless-steel (SS) and polypropylene B (PP) coupons, in three culture media to simulate the conditions in the dairy, meat, and egg processing industries, at 9 and 25 °C.

2. Materials and methods

2.1. Bacterial strains and culture media

The microorganisms used in biofilm formation were *E. coli* ATCC 11303, *S.* Typhimurium ATCC 14028, *S.* Enteritidis ATCC 13076, *P. aeruginosa* ATCC 15442, *L. monocytogenes* ATCC 19111, and *B. cereus* ATCC 14579. These bacteria can develop biofilms and represent potential foodborne pathogens and spoilage microorganisms in the dairy, meat, and egg processing industries (Motarjemi and Lelieveld, 2014). Before utilization, the microorganisms were incubated individually in tryptic soy broth (TSB; Becton Dickinson Bioxon, Le Pont de Claix, France) at 37 °C for 24 h, to yield a final concentration of 10^7 CFU/mL.

2.2. Biofilm development

2.2.1. Contact surfaces

The SS (AISI 304, 2 × 1 × 0.1 cm; CIMA Inoxidables, Jalisco, Mexico) and PP coupons (2 × 1 × 0.2 cm; Plásticas Tarkus, Jalisco, Mexico) were cleaned, according to the method of Rossoni and Gaylarde (2000), modified by Marques et al. (2007). Briefly, the surfaces were immersed in pure acetone (Fermont, Monterrey, Nuevo León, Mexico) for 1 h, to remove any debris and grease, immersed in neutral detergent (30 mL/L; Cip & Group S. de R.L., Tlajomulco de Zuñiga, Jalisco, Mexico) for 1 h, rinsed with sterile distilled water, cleaned with ethanol (70%; Hycel, Zapopan, Jalisco, Mexico), dried for 2 h at 60 °C, and sterilized by autoclaving (121 °C for 15 min).

2.2.2. Development of mono- and multispecies biofilms and quantification

Biofilms were developed in three culture media: TSB with chicken egg yolk (TSB + EY, 10%), TSB with meat extract (TSB + ME, 10%; Becton Dickinson Bioxin), and whole milk (WM) processed at ultra-high temperature was purchased from a retail shop in Jalisco (Mexico). Each coupon was individually introduced into a new polypropylene tube (15 mL Centrifuge Tube; Corning CentriStar) containing 5 mL of the corresponding culture media. The monospecies biofilms were inoculated with 25 μL (1 × 10^6 CFU/mL) of the corresponding strain, the tubes were incubated for 240 h at 9 or 25 °C. For multispecies biofilms, each tube was inoculated with 25 μL of each bacterial species (1 × 10^6 CFU/mL); *S.* Typhimurium was used in biofilms developed in TSB + ME and WM, and *S.* Enteritidis was inoculated in TSB + EY. The tubes with the coupons were incubated at 9 or 25 °C for 1, 48, 120, 180, and 240 h. At 120 h incubation, the coupons were removed from the tube and immersed into a fresh medium containing the same microorganism concentration (1 × 10^6 CFU/mL); and were incubated to complete 240 h (Heydorn et al., 2000; Xu et al., 2010). After each incubation period, the coupons were removed from the tube, under sterile conditions, and rinsed by vortexing (150 rpm for 10 s) in 5 mL of Dulbecco's phosphate-buffered saline (Sigma–Aldrich) to remove the loosely attached cells (Kostaki et al., 2012). Each coupon was introduced individually into 3 mL of casein peptone (1 g/L; Becton Dickinson Bioxon), and the biofilms were removed by sonication (50–60 Hz for 1 min; Sonicor Model SC-100TH, West Babylon, NY, USA). Serial dilutions and conventional plate counting on tryptic soy agar (Becton Dickinson Bioxin) with lactose (10 g/L; Sigma–Aldrich) and phenol red (0.1 g/L; Hycel, Zapopan, Jalisco, Mexico) were realized. For quantification of *E. coli* and *B. cereus* in multispecies biofilms, cefsulodin (50 μg/mL; Sigma–Aldrich) and polymyxin B (70 μg/mL; Sigma–Aldrich) were added, respectively. Petri dishes were incubated at 37 °C for 24 h (Mariscal et al., 2009). Colonies of *E. coli* and *L. monocytogenes* were yellow, due to lactose fermentation and the remaining microorganism colonies were colorless. *Salmonella* and *P. aeruginosa* were distinguished based on the oxidase test. Each quantification was conducted in triplicate. A control without microorganisms was included for determination of any contamination.

2.3. Microscopy analysis

2.3.1. Epifluorescence microscopy

After each incubation period (1, 48, 120, 180, and 240 h), the mono- and multispecies biofilms were stained with 5(6)-carboxyfluorescein diacetate (CFDA, 10 μg/mL), and dried in a level II cabinet; the CFDA excess was rinsed with sterile distilled water. The coupons were examined under a Nikon Eclipse E400 epifluorescence microscope, using 100× oil immersion lens and a BA 515 B-2[a] filter, at 450–490 nm and at least 15 fields were observed (Gorokhova et al., 2012).

2.3.2. Scanning electron microscopy (SEM) analysis

After each incubation period (as above), a coupon of each material

was removed from the tube, rinsed with Dulbecco's phosphate-buffered saline, and immersed in 2% glutaraldehyde at 4 °C for 2 h, to fix the adherent bacteria (Alhede et al., 2012; Meira et al., 2012). After serial dehydration in ethanol (30, 50, 60, 70, 90, and 95%) for 10 min each, at 4 °C, every coupon was rinsed (three 10-min rinses) in 100% ethanol (Borucki et al., 2003). Samples were critical-point dried and were coated with gold for 30 s (Fratesi et al., 2004). The biofilms were viewed under a Mira 3 LMU field emission scanning electron microscope (Tescan, Czech Republic).

2.4. Statistical analysis

All experiments were performed in triplicate and the data evaluated using analysis of variance (ANOVA), followed by a least significant difference (LDS) test, in the Statgraphics Centurion XV software (Statpoint Technologies, Inc., Warrenton, VA, USA).

3. Results

3.1. Monospecies biofilms

The cell density in the biofilms and cellular viability were evaluated using standard plate counting and epifluorescent microscopy. All the tested microorganisms showed a high ability to develop biofilms in the three culture media reaching > 7.70 Log_{10} CFU/cm^2 (Fig. 1). In TSB + ME, *P. aeruginosa* not had a significative difference ($p > 0.05$) in the cell density under the tested conditions, however; *E. coli* and *B. cereus* had differences in their populations in each condition ($p < 0.05$) (Fig. 1A). On the other hand, in TSB + EY *L. monocytogenes* had the lower density (7.9 Log_{10} CFU/cm^2) in comparison to the other microorganisms, while *B. cereus* had the higher densities in comparison to its population in TSB + ME and WM (Fig. 1B). Finally, in WM, *P. aeruginosa* had the lower density (8.03 Log_{10} CFU/cm^2) ($p < 0.05$) in comparison to the other microorganism in the same media, except that *L. monocytogenes* on PP at 9 °C (8.12 Log_{10} CFU/cm^2) ($p > 0.05$) (Fig. 1C). In monospecies biofilms was not observed significative influence of surface type or temperature on all tested microorganisms ($p > 0.05$), the conditions had a different effect in the biofilm development by each microorganism (Fig. 1).

3.2. Tryptic soy broth with meat extract (TSB + ME)

The bacterial amounts in biofilms are summarized in Fig. 2. At 1 h incubation in TSB + ME, each bacterial species oscillated between 1.43 (*B. cereus*) and 6.08 Log_{10} CFU/cm^2 (*P. aeruginosa*) (Fig. 2C, D). On PP at 25 °C, *P. aeruginosa*, *S.* Typhimurium, *E. coli*, *L. monocytogenes*, and *B. cereus* reached 6.08, 5.22, 3.63, 2.63, and 1.43 Log_{10} CFU/cm^2 ($p < 0.05$) (Fig. 2C), respectively. At this stage, *B. cereus* was not detected (Fig. 2B). Even when all biofilms were inoculated with the same amount of each bacteria (7.33 ± 0.14 Log_{10} CFU/mL), statistical differences were observed in the cell density of each microorganism (Fig. 2). In the reversible attachment, the Gram-negative bacteria reached the highest densities (Fig. 2A–D).

The detachment phase is characterized by the decrement in cell counts. In TSB + ME, there were significant differences between the counts at 1 and 48 h, in biofilms developed at 9 °C, for *S.* Typhimurium on SS (Fig. 2B), and *B. cereus* and *E. coli* on PP ($p < 0.05$) (Fig. 2D). Instead, *L. monocytogenes* showed a linear growth during the 240 h incubation, with increments of 3.18 and 3.87 Log_{10} CFU/cm^2 on PP at 9 °C (Fig. 2D). However, after incubation at 25 °C for 48 h, it displayed an increase of 2.35 Log_{10} CFU/cm^2 (Fig. 2C) on PP and an even lower increment of 0.68 Log_{10} CFU/cm^2 on SS (Fig. 2A). Otherwise, *S.* Typhimurium, *P aeruginosa*, and *L. monocytogenes* remained constant since 48 h incubation at 25 °C on both surfaces (Fig. 2A, C). A significant decrement was noticed for *P. aeruginosa* on PP at 9 °C at 240 h, related to biofilm dispersion (Fig. 2D). At the end of the incubation period, the

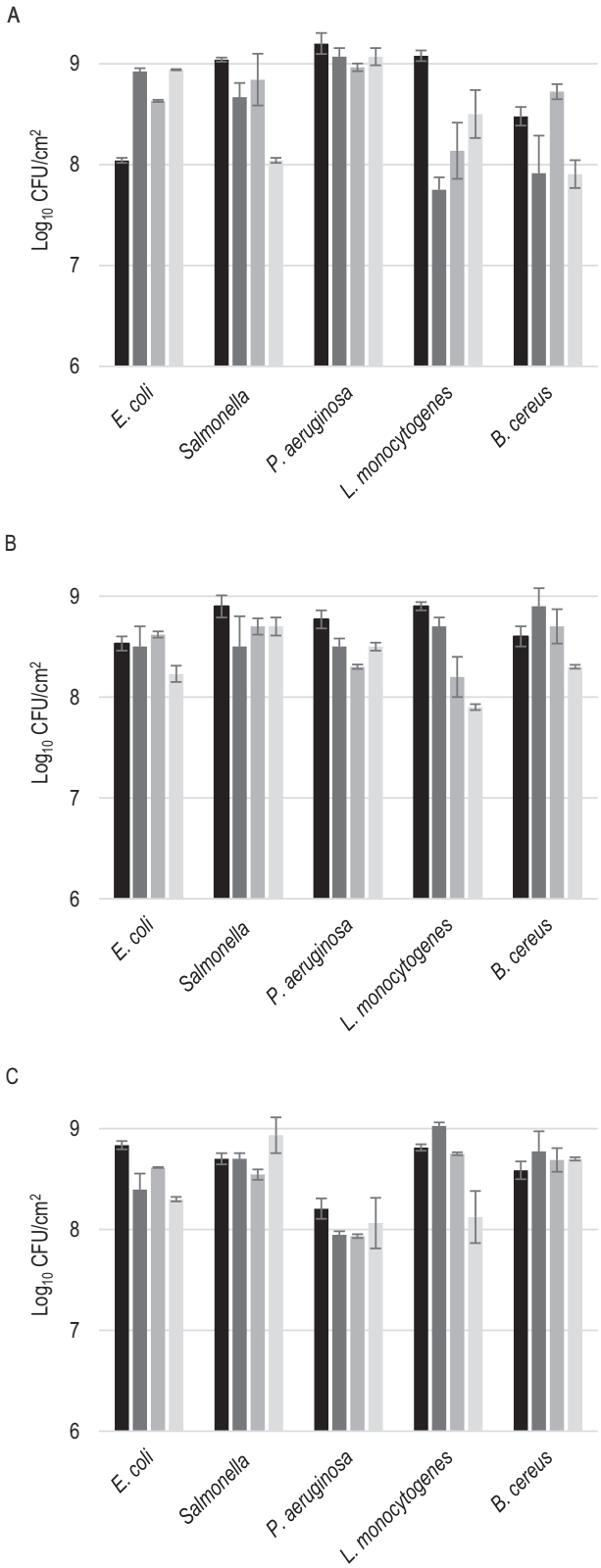

Fig. 1. Cellular density in monospecies biofilms. The biofilms ere developed onto stainless-steel (SS) and polypropylene B (PP), in A) tryptic soy broth (TSB) with 10% meat extract, B) TSB with 10% egg yolk and C) whole milk and were incubated at 9 and 25 °C during 240 h. SS at 25 °C (■), PP at 25 °C (■), SS 9 °C (■) and PP 9 °C (■). Each bar represent the mean of three test ± standard deviation of the means (n = 3). Detection limits were 0.71 and 0.81 Log_{10} CFU/cm^2 to PP and SS, respectively.

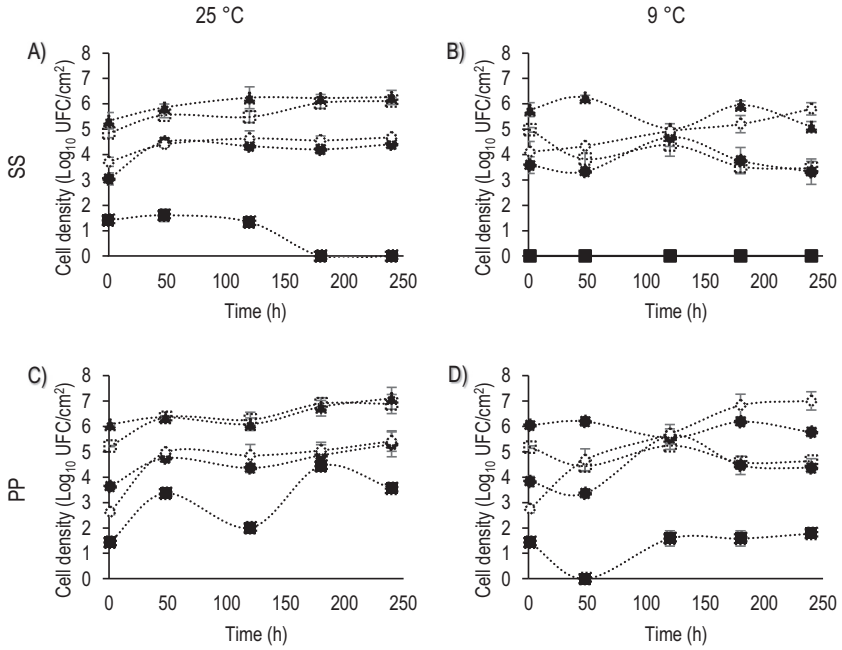

Fig. 2. Cell counts in multispecies biofilms developed in tryptic soy broth with meat extract (TSB + ME). The biofilms were developed on stainless-steel (SS) and polypropylene B (PP), cellular densities were determined at five times (1, 48, 120, 180, and 240 h). A) SS at 25 °C; B) SS at 9 °C; C) PP at 25 °C; D) PP at 9 °C. *Escherichia coli* ATCC 11303 ●; *Salmonella* □; *Pseudomonas aeruginosa* ATCC 15442 ▲; *Listeria monocytogenes* ATCC 19111 ◊; *Bacillus cereus* ATCC 14579 ■. Vertical bars represent the standard deviations of the means (n = 3). Detection limits were 0.71 and 0.81 Log_{10} CFU/cm^2 to PP and SS, respectively.

Table 1
Cell density in multispecies biofilms after 240 h of incubation in different culture media.

Culture media	Cell density in multispecies biofilms (CFU/cm^2)[a]			
	Stainless steel		Polypropylene	
	9 °C	25 °C	9 °C	25 °C
TBS + ME	5.87 ± 0.15 A[b]a[c]	6.46 ± 0.20 Ab	6.95 ± 0.23 ABc	7.18 ± 0.45 Ac
TBS + EY	5.78 ± 0.12 Aa	6.67 ± 0.09 Ab	7.19 ± 0.34 Bc	7.07 ± 0.28 Ac
WM	5.09 ± 0.18 Ba	6.55 ± 0.27 Ab	6.59 ± 0.07 Cb	7.91 ± 0.07 Bc

[a] Mean of three test of population in multispecies biofilms Log_{10} ± standard deviation of the means (n = 3).
[b] Values in the same column with the same capital letter are not significantly different ($p > 0.05$).
[c] Values in the same row with the same lowercase letter are not significantly different ($p > 0.05$).

cell density in multispecies biofilms developed was higher onto PP than on SS; the temperature had a significative effect in cell density in the biofilms, being higher at 25 °C than at 9 °C (Table 1).

TSB + ME: tryptic soy broth with 10% meat extract; TSB + EY: tryptic soy broth with 10% egg yolk and WM: whole milk.

3.3. Tryptic soy broth with egg yolk (TSB + EY)

Results of multispecies biofilms developed on SS and PP in TSB + EY are shown in Fig. 3. After incubation at 9 °C for 1 h on PP, the amount of each species was between undetectable levels (*B. cereus*) to 5.70 Log_{10} CFU/cm^2 (*P. aeruginosa*), with intermediate counts of 3.36 (*L. monocytogenes*), 3.65 (*E. coli*), and 4.97 Log_{10} CFU/cm^2 (*S.* Enteritidis) (Fig. 3D). A decrease of 1 Log_{10} CFU/cm^2 in *P. aeruginosa* occurred after incubation on SS at 9 °C for 48 h ($p < 0.05$) (Fig. 3B). The linear growth phase presented mainly at 25 °C between 48 and 180 h, with increments of 0.9 and 1.27 Log_{10} CFU/cm^2 for *P. aeruginosa* (Fig. 3C) and *S.* Enteritidis (Fig. 3A), respectively. Moreover, *L. monocytogenes* exhibited a linear growth throughout the 240 h incubation, evidencing an increment of 3.87 Log_{10} CFU/cm^2 on PP at 9 °C (Fig. 3D). The tested microorganisms established cell population from 120 h incubation at 9 °C on SS ($p > 0.05$) (Fig. 3B). Biofilm dispersion was observable for *B. cereus*, *S.* Enteritidis and *E. coli* in the biofilms developed at 9 °C on PP, with cell reduction rates of 1.22, 1.55, and 1.59 Log_{10} CFU/cm^2, respectively, in comparison with the densities obtained at 120 h incubation ($p < 0.05$) (Fig. 3D). After 240 h of incubation, the cell density was higher onto PP than on SS; the temperature had a significative effect in biofilm development on both surfaces (Table 1).

3.4. Whole milk (WM)

After 1 h incubation, the cell density of each microorganism in biofilms developed in WM oscillated between 2.41 and 5.86 Log_{10} CFU/cm^2 (Fig. 4), this variation was less in comparison with that shown the biofilms developed in TSB + ME (Fig. 2) and TSB + EY (Fig. 3) ($p < 0.05$). For these biofilms at 25 °C, *B. cereus* was detected only after incubation for 1 h, on both SS and PP surfaces (Fig. 4A, C). Significant decrements in *S.* Typhimurium, *B. cereus*, and *E. coli* were apparent on both surfaces at 9 °C (Fig. 4B, D) but the highest decrements (1.12 Log_{10} CFU/cm^2) corresponded to *B. cereus* on PP at 9 °C, and until undetectable levels in the others tested conditions (Fig. 4A–C). There was a linear growth in *L. monocytogenes* during the 240 h of incubation, with increments of 2.98 Log_{10} CFU/cm^2 at 25 °C in WM, on SS (Fig. 4A). The other microorganisms, like *S.* Typhimurium, increased 2.08 Log_{10} CFU/cm^2 between 48 and 180 h, on SS (Fig. 4A). In WM at 25 °C, the microorganisms maintained their densities on SS from 48 h, except *S.* Typhimurium, which was stable at 180 h. However, on PP, the populations were stable from 180 to 240 h ($p > 0.05$) (Fig. 4A, C). At the end of incubation on SS at 9 °C, all the microorganisms in the biofilm demonstrated reductions between 1.2 and 2.80 Log_{10} CFU/cm^2 ($p < 0.05$), except *L. monocytogenes*, which maintained its population ($p > 0.05$) (Fig. 4B). In WM, at the end of the incubation period, the

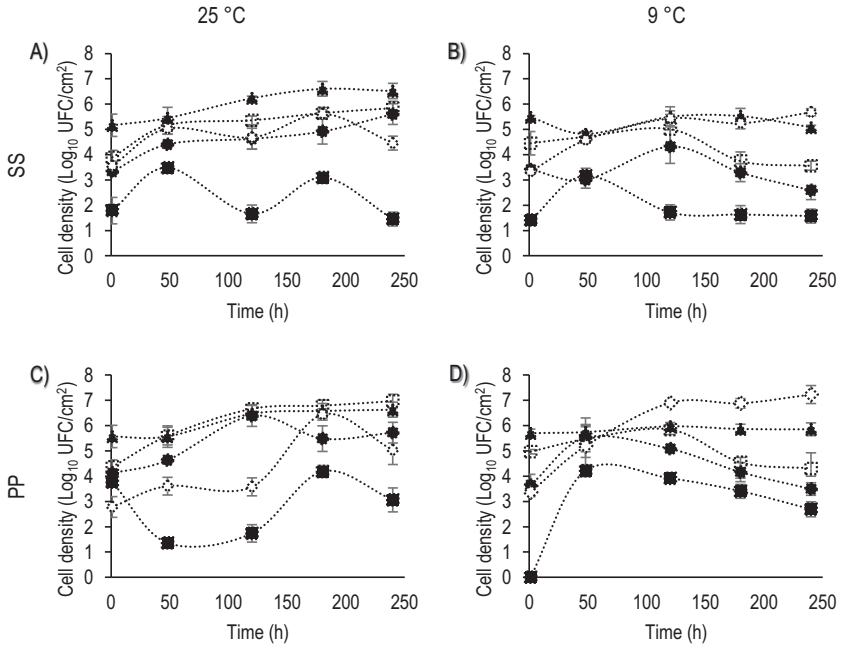

Fig. 3. Cell counts in multispecies biofilms developed in tryptic soy broth with egg yolk (TSB + EY). The biofilms were developed on stainless-steel (SS) and polypropylene B (PP), cellular densities were determined at five times (1, 48, 120, 180, and 240 h). A) SS at 25 °C; B) SS at 9 °C; C) PP at 25 °C; D) PP at 9 °C. *Escherichia coli* ATCC 11303 ●; *Salmonella* □; *Pseudomonas aeruginosa* ATCC 15442 ▲; *Listeria monocytogenes* ATCC 19111 ◊; *Bacillus cereus* ATCC 14579 ■. Vertical bars represent the standard deviations of the means (n = 3). Detection limits were 0.71 and 0.81 Log_{10} CFU/cm^2 to PP and SS, respectively.

temperature had a significative effect in the cell density in the biofilms on both surfaces; however, the population was higher on PP than in the same tested condition onto SS (Table 1).

The microorganisms that predominate in the biofilms were *P. aeruginosa* and *Salmonella*. It is also important to mention that the increase shown by the microorganisms at 180 h compared to 120 h, and the significant decrease at 240 h, could be associated with the re-inoculation and the replacement of the culture medium (Figs. 2B, C, 3A, C and 4B).

3.5. Epifluorescence and scanning electron microscopy observations

Representative images of monospecies biofilms on SS and PP were obtained using epifluorescence microscope (Fig. 5). In all the cases metabolically-active cells were observed. Multispecies biofilms were observed in different development stages on SS coupons. In this biofilms, besides active cells, the accumulation of extracellular polymeric substances (EPS) and food residues were observed. The amount of polymeric matrix increasing during the incubation time, for this was more difficult to observe the cells in the biofilms developed in TSB + EY and WM. After 1 h incubation few cells were observed, but according to the increment in the incubation time, the number of cells increased. Additionally, more cell aggregation was observed in biofilms developed at 25 °C than those developed at 9 °C (Fig. 6). Trough SEM verified these observations. Biofilms developed in WM showed increments of microorganisms and EPS a long incubation time. Additionally, higher amounts of bacteria were observed on PP than SS, in concordance with the plate count results (Fig. 7). Surfaces covered with the organic matter were also noticed (preconditioned surfaces, Fig. 7B, I).

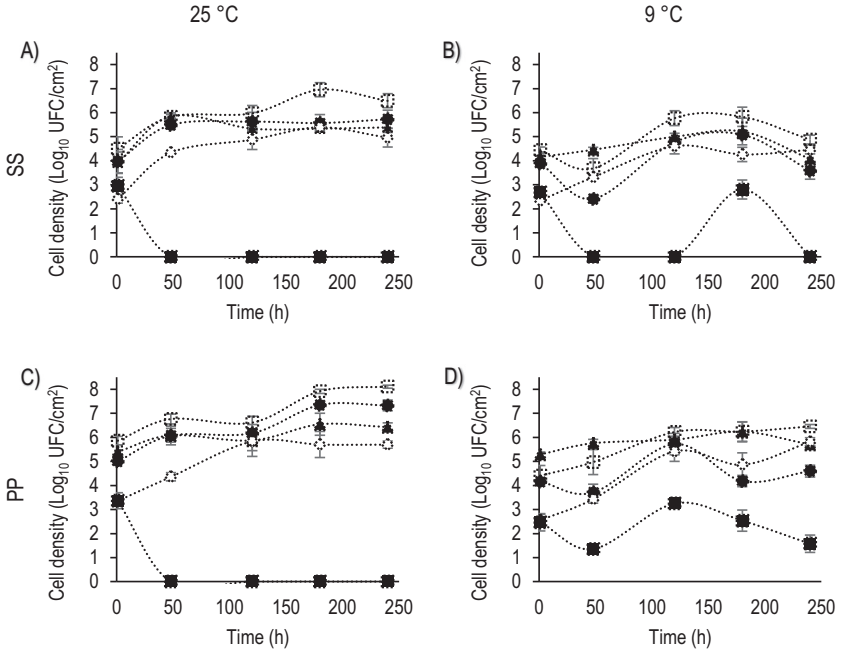

Fig. 4. Cell counts in multispecies biofilms developed in whole milk (WM). The biofilms were developed on stainless-steel (SS) and polypropylene B (PP), cellular densities were determined at five times (1, 48, 120, 180, and 240 h). A) SS at 25 °C; B) SS at 9 °C; C) PP at 25 °C; D) PP at 9 °C. *Escherichia coli* ATCC 11303 ●; *Salmonella* □; *Pseudomonas aeruginosa* ATCC 15442 ▲; *Listeria monocytogenes* ATCC 19111 ◊; *Bacillus cereus* ATCC 14579 ■. Vertical bars represent the standard deviations of the means (n = 3). Detection limits were 0.71 and 0.81 Log_{10} CFU/cm^2 to PP and SS, respectively.

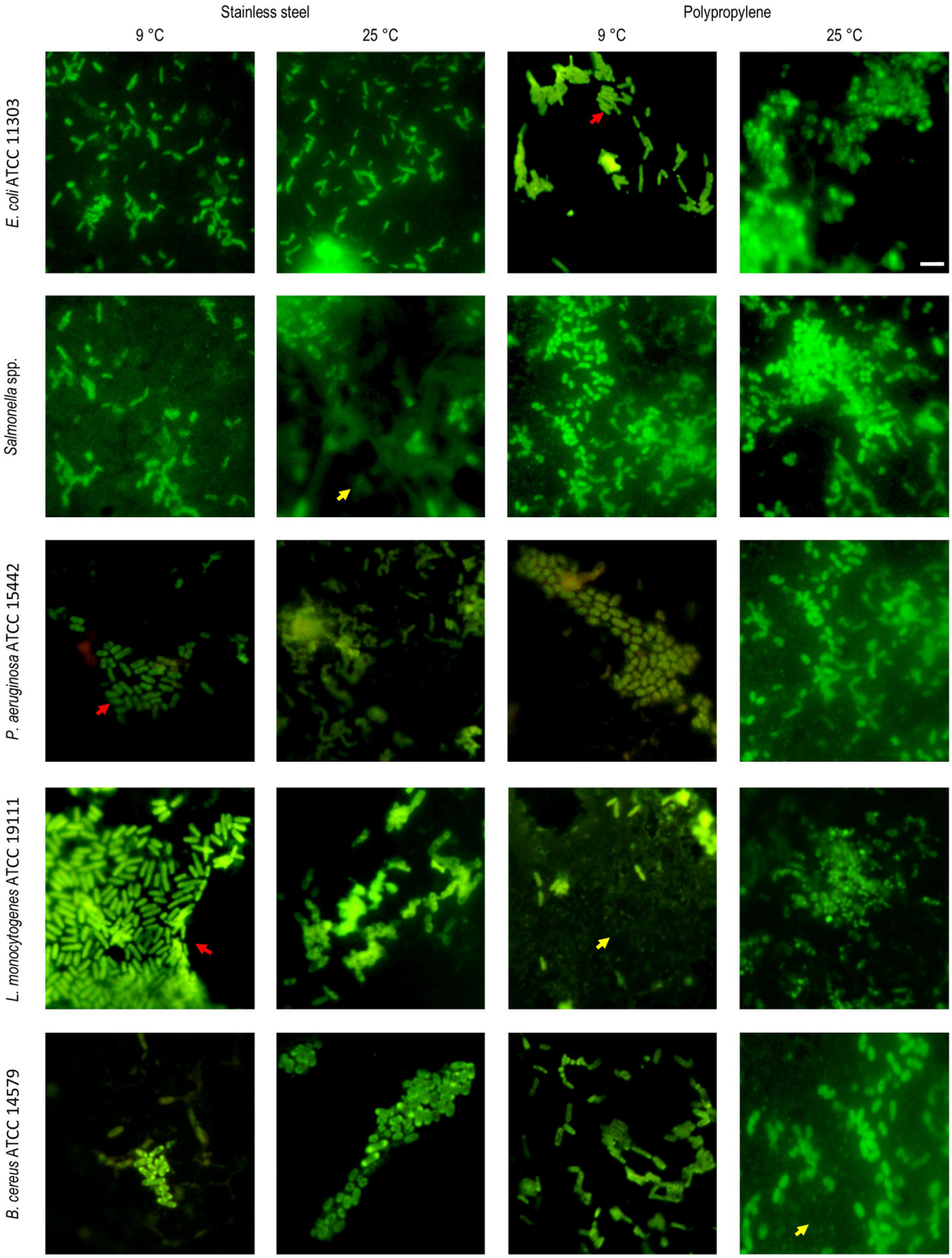

Fig. 5. Micrographs of monospecies biofilms development onto stainless-steel (SS) and polypropylene B by epifluorescence microscopy. The biofilms were developed at 9 and 25 °C for 240 h in tryptic soy broth (TSB) with 10% meat extract. The white bar scale indicates 5 μm. The red arrows shown the presence of metabolically-active cells, whereas the yellow arrows indicates the presence of EPS and food residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

In the food industry, foods residues are the main source used by the microorganisms to growth (Lamas et al., 2018). It is therefore of great importance to create growth environments in research laboratories that mimic actual food industry conditions (Li et al., 2017). All the monospecies biofilms had a higher cellular density in comparison with the multispecies biofilms ($p < 0.05$), this is related with the ability of each strain to develop biofilms in mixed culture (Iñiguez-Moreno et al., 2018; Kostaki et al., 2012). To this the kinetics of biofilms formation under conditions that mimic the dairy, meat and egg processing industry were tested onto SS and PP. After 1 h incubation, high bacterial

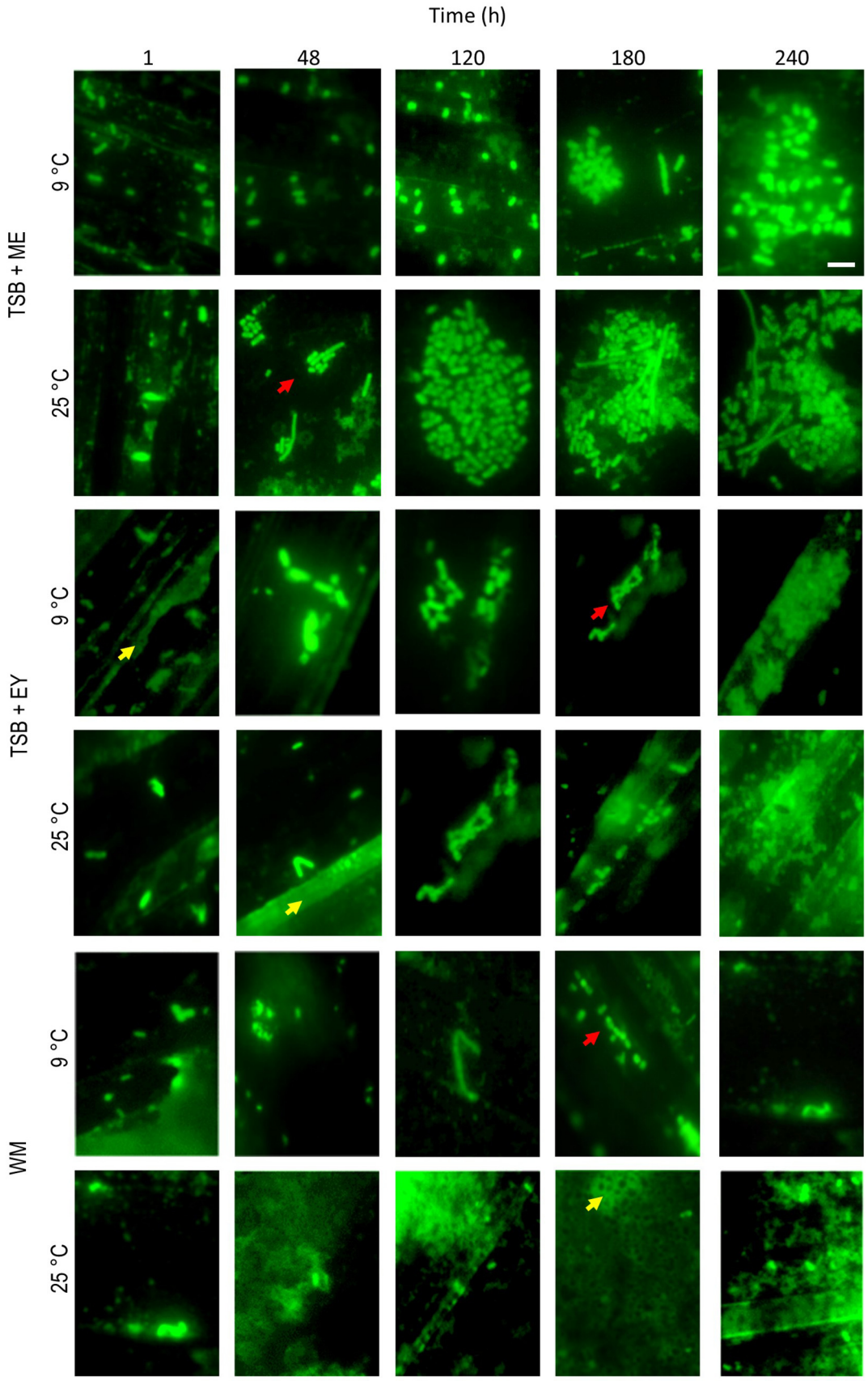

Fig. 6. Micrographs of multispecies biofilms development on stainless-steel (SS) by epifluorescence microscopy. The biofilms were developed at 9 and 25 °C for 240 h in tryptic soy broth (TSB) with 10% meat extract, TSB with 10% egg yolk and whole milk, with a replacement of culture medium and re-inoculation of the microorganisms at 120 h. The white bar scale indicates 5 μm. The red arrows shown the presence of metabolically-active cells, whereas the yellow arrows indicates the presence of EPS and food residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

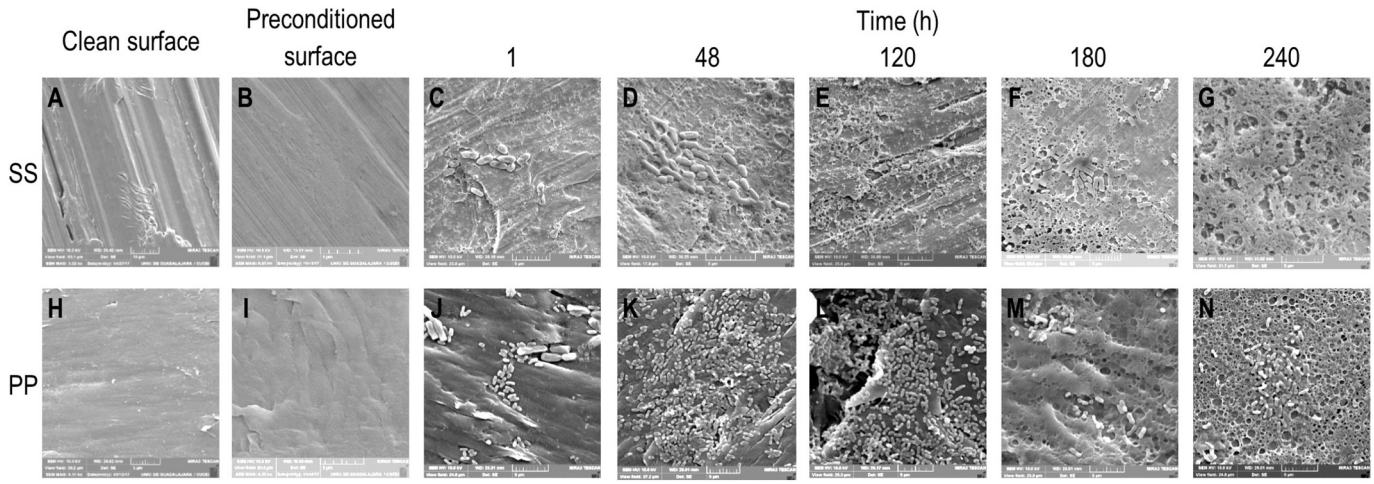

Fig. 7. Micrographs of multispecies biofilm formation stages in whole milk. The biofilms were developed by *Escherichia coli* ATCC 11229, *Salmonella* Typhimurium ATCC 14028, *Pseudomonas aeruginosa* ATCC 15442, *Listeria monocytogenes* ATCC 19111, and *Bacillus cereus* ATCC 14579, at 25 °C on stainless-steel (SS) and polypropylene B (PP). The biofilm formation was observed at 1, 48, 120, 180, and 240 h incubation.

counts were obtained in all tested conditions, which could be related to the previous step in the biofilm formation, namely, the surface preconditioning (Dat et al., 2014). In this step, the particles of the medium coat the surface and modify its charge, increasing bacterial adherence (Chmielewski and Frank, 2003). The surfaces adsorb milk and milk components (caseins, β-lactoglobulin, α-lactalbumin, and serum albumin) within the first 5 to 10 s of contact (Dat et al., 2014; Mittelman, 1998), facilitating bacterial adherence (Kuda et al., 2015). Likewise, the presence of lipopolysaccharides (in Gram-negative bacteria) and bacterial appendages (adhesins, fimbriae, and flagella) have been implicated in cell aggregation and biofilm formation, by reducing the repulsion forces (Bridier et al., 2015; Steenackers et al., 2012). Also, flagella contribute to biofilm spread (Van Houdt and Michiels, 2010).

The desorption phase is a decrement in the cell count during sample processing compared with initial colonization (Xu et al., 2010). In this research, there was a significant difference between the counts obtained at 1 and 48 h, in biofilms developed at 9 °C. Xu et al. (2010), found that pathogenic bacteria showed this phase in TSB with low levels of NaCl (0, 2, and 4%), but not in biofilms developed with high NaCl (6, 8, and 10%), affirming that this phase is dependent on environmental conditions and microorganism type. Meira et al. (2012) evaluated the desorption kinetics of *S. aureus* by contact with agar and noted a cell detachment of around 3 Log_{10} CFU/cm^2, which is relatively higher than our results, but this difference is explained by the mechanical forces applied. Additionally, after 48 h of incubation in TSB + ME *E.coli* reached 4.62 Log_{10} CFU/cm^2 while it has been reported that in trispecies biofilms with *L. monocytogenes* and *S. enterica* after the same time *E. coli* had a cell density of 8.32 Log_{10} CFU/cm^2 (Almeida et al., 2011). This could be associated with the antagonistic effect of extracellular peptides secreted by *P. aeruginosa* (Ma et al., 2009) and the bacteriocin produced by *B. cereus* ATCC 14579 (Risøen et al., 2004); and to the use of meat extract.

In linear growth phase, irreversible adhesion process begins, cell-cell interactions allow the establishment of complex and highly structured biofilms (Giaouris et al., 2015). *L. monocytogenes* showed a large linear phase in biofilms developed at 9 °C, attributable to its psychrotrophic nature (Lee et al., 2014); conversely, it has been reported that *L. monocytogenes* density in multispecies biofilms tends to be low, because their EPS production is low (Borucki et al., 2003); nonetheless *Pseudomonas* spp. can increase its colonization and persistence on surfaces (Fox et al., 2014; Giaouris et al., 2015).

The stabilization and maturation phases are characterized by cellular densities maintenance at different times, *S.* Enteritidis showed cell densities of 5.03 and 5.64 Log_{10} CFU/cm^2 ($p < 0.05$) at 48 and 180 h respectively, on SS at 25 °C in TSB + EY (Fig. 3A). This result is greater than reported by Yang et al. (2017) who developed *S.* Enteritidis ATCC 13076 monospecies biofilms on SS AISI 304 at 25 °C in TSB with 10% EY; and obtained 4.81 Log_{10} CFU/cm^2 after 48 of incubation. Therefore, in the current study, the interspecies interactions contributed to increase the cellular density of *S.* Enteritidis onto SS in TSB + EY. In addition, the expression of the red, dry and rough morphotype (*rdar*) linked to cellulose production (also produced by *E. coli*) is favored at 25–28 °C under aerobic conditions, during the stationary phase when cell density is high, and microorganisms compete for space and nutrients (Lamas et al., 2018). During these stages, proteases and lipases are synthesized by *P. aeruginosa* and *B. cereus* (Pinto et al., 2010; Ribeiro et al., 2015); the use of meat, milk and egg yolk proteins as substrates lead to a decrease in the growth rate and an increase in the pH of the medium caused by deamination (Mellefont et al., 2008). This contributes to microbial dispersion and the return to planktonic lifestyle, to reach sites rich in nutrients and colonize other surfaces (O'Toole et al., 2000; Srey et al., 2013).

Cell density decrements were mainly showed by *L. monocytogenes* and *B. cereus* in biofilms at 25 °C in TSB + EY (Fig. 3A, C); in WM at 9 °C on SS all microorganisms in the biofilm showed this phase except *L. monocytogenes* (Fig. 4B). Moreover, biofilm dispersion is related to an increase of secondary metabolites and oxygen depletion (O'Toole et al., 2000; Srey et al., 2013); this stage is commonly observed in biofilms that have been developed in a nutrient-rich environment (Flemming et al., 2011).

Salmonella and *P. aeruginosa* were typically present in higher amounts than the other tested microorganisms; this can be associated with their ability to use the media components linked the interspecies interactions (Kostaki et al., 2012); moreover, predominant microorganisms in the biofilms are those with a higher growth rate, although these can never completely exclude the microorganisms with slow growth (Banks and Bryers, 1991).The low counts of *B. cereus* in all tested biofilms could be related with the interspecies interactions (Chorianopoulos et al., 2008; Lebert et al., 2007). The bacteriostatic or bactericidal responses can be attributed to metabolic products with antagonistic effects (organic acids, inhibitory enzymes, and others), physicochemical changes, nutrient depletion or combination of these (Mellefont et al., 2008; van der Veen and Abee, 2011). Additionally, *Pseudomonas* spp. compete efficiently to iron and oxygen causing the suppression of other microorganisms (Tiwari et al., 2016) and has growth velocity until 10 times higher at 7 °C than other bacteria (Ribeiro et al., 2015).

In monospecies biofilms, the surface had no effect in cell density

($p > 0.05$), due to exist others factors implicated in the biofilm development, such as nutrients and temperature; moreover it has been reported that microorganisms had the same ability to develop biofilms on hydrophilic or in hydrophobic surfaces (Iñiguez-Moreno et al., 2018). However, the multispecies biofilms had a higher population on PP than on SS due to the SS is a hydrophilic material with metallic ions, these factors contributing to limit the bacterial adhesion, mean the PP is a hydrophobic surface (Das, 2014; Schlisselberg and Yaron, 2013). So these results suggest that the surface finish should be carefully considered when designing a food processing plant, since the surface should be as smooth as possible so that the shearing force increases.

The use of epifluorescence microscopy permits observing metabolically-active living cells. Intracellular non-specific esterases hydrolyze the internalized CFDA, producing carboxyfluorescein, which is retained by live cells with an intact plasma membrane. Hence, the conversion to carboxyfluorescein by the cells indicates the integrity of the plasma membrane and the esterase activity (Gorokhova et al., 2012). Although SS and PP are not autofluorescence materials at the range used (450–490 nm) (Jun et al., 2010), TSB and EY residues emit fluorescence (Lee et al., 2008; Yang et al., 2017), explaining the difficulty in discerning the bacterial cells in the last biofilm formation stages. Moreover, the biofilms are complex structures conformed by multilayers of microorganisms, EPS and water channels (Hobley et al., 2015). Although SEM, which is one of the most common methods for the study of biofilms, enables observing the biofilms architecture (EPS and bacteria) and development, it visualizes both living and dead cells (Alhede et al., 2012), so for biofilm studies, complementary techniques should be used.

5. Conclusion

Pathogens and spoilage microorganisms developed mono- and multispecies biofilms under all tested conditions; however, the cell density was higher in monospecies biofilms. The type of surface had not significative effect on monospecies biofilms, but the biofilm development by multiple microorganisms was favored on PP than onto SS; additionally, at 25 °C increase the biofilm development on both surfaces. In multiespecies biofilms, the time to reach each biofilm step varied; this variation is attributable to the influence of the culture media, temperature, surface type, and the growth of the microorganisms in the biofilms. Desorption and dispersion phases of biofilm formation were most affected by the mentioned factors. Moreover, *P. aeruginosa* and *Salmonella* were the dominant microorganisms in the biofilms while *B. cereus* showed the lowest densities. Hence in the food industry, special attention should be pay first in the selection of the appropriated food contact surface and then in the management of cleaning and sanitization programs, with the aim of avoiding the food residues accumulation reducing the bacterial attachment.

Declaration of Competing Interest

None.

Acknowledgments

The authors gratefully acknowledge Cip & Group S. de R.L for the financial assistance, and particularly, Ing. Jesús Casillas (General Director) and Ing. Sergio Miramontes (Technical Assessor); and thank the National Council of Science and Technology of Mexico, for the scholarship awarded to Maricarmen Iñiguez.

Funding

This research was supported by Cip & Group S. de R.L; and through a scholarship granted to Maricarmen Iñiguez by the National Council of Science and Technology of Mexico [grant number 404464].

Ethical approval

Not required.

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