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Mono- and Mixed-Species Biofilm Formation by *Salmonella* Infantis, *Salmonella* Kentucky, *Enterococcus faecium*, and *Enterococcus faecalis*

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Abstract

It is crucial to understand the interactions between foodborne pathogens, as they are commonly encountered as multi-species biofilms in the food industry. Since *Salmonella* and *Enterococcus* are both found in poultry intestinal microbiota, the multi-species biofilms of these strains gain more significance. This study aimed to reveal the synergistic and antagonistic effects of mono- and mixed-species biofilms of *Salmonella Infantis, Salmonella* Kentucky, *Enterococcus faecium*, and *Enterococcus faecalis* on each other. Biofilm formation of mono- and mixed-species at different concentrations (10^9, 10^7, 10^5, and 10^3 cfu/mL) were determined in Tryptic Soy Broth using polystyrene microplates at room temperature (21 ± 1°C) for 48 hours. According to the optical density measured at 590 nm, *S. Infantis* was determined as a strong biofilm producer, and *S.* Kentucky and *E. faecium* were defined as weak biofilm producers under the conditions applied in the study. No biofilm formation was observed in *E. faecalis*. In addition, while *S. Infantis* and *S.* Kentucky exhibited an antagonistic effect on each other when co-incubated, a synergistic effect was seen between the *S. Infantis* and *Enterococcus* spp. This study highlights the impact of microbial interactions in mixed-species biofilm formed by foodborne pathogens, and could help in future studies on combating biofilms, especially in poultry-based food processing environments.

Keywords: Biofilm, *Enterococcus*, mixed-species, mono-species, *Salmonella*

Introduction

Biofilm, matrix-embedded bacterial populations that adhere to a surface or to each other, allows bacteria to survive in adverse environmental conditions (Hall-Stoodley et al., 2004). Biofilms formed by pathogenic microorganisms play a major role in food contamination and can become resistant to sanitation products. It is stated that a large amount of money is spent each year to deal with human infections caused by food contamination, equipment damage, and biofilms (Elexson et al., 2014; Yang et al., 2011).

Salmonellosis remains one of the most frequent foodborne zoonoses, creating a worldwide major public health concern. *Salmonella* has the capability to adhere to surfaces such as glass, plastic, and stainless steel, and form biofilms. When formed on contact surfaces, biofilms can be a permanent source of contamination and can have serious effects on public health. Besides, increasing numbers of infections by *Salmonella* Kentucky and *Salmonella Infantis* due to poultry meat are becoming more common (Antunes et al., 2016). According to a study (Diker et al., 2020) in which the results were obtained from a project entitled "Development of Control and Monitoring Program for *Salmonella* in Poultry and Poultry Products," *S. Infantis* was found as the dominant serotype in broilers, breeder flocks, slaughterhouses, turkey meat, and food samples, while *S.* Kentucky was reported as the predominant serotype detected in all of the sample matrices collected from laying hens in Turkey.

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Enterococci are commonly found in the intestinal microbiota of animals and humans. *Enterococcus faecalis* and *Enterococcus faecium* are the dominant species that can be isolated from the stool of healthy individuals (Landete et al., 2018), chickens during slaughtering (Kasimoglu-Dogru et al., 2010), and dairy products (Buyukyoruk et al., 2013). While commensal *Enterococcus* spp. do not cause any pathogenic impacts on healthy individuals in general, they can cause gastrointestinal and urinary system damage and even endocarditis in susceptible hosts (Da Silva Fernandes et al., 2017; García-Solache & Rice, 2019). Because of their high tolerance to various ambient conditions like excessive concentrations of salt, temperature, and a wide range of pH, enterococci are associated with biofilms (Arias & Murray, 2012; Ch'ng et al., 2019). Biofilm production increases the intrinsic resistance of enterococci to antibiotics. It is also reported that enterococci can transfer the antibiotic resistance genes of biofilms horizontally (Dale et al., 2015; La-Rosa et al., 2016). On the other hand, the biofilm formed by enterococci makes it difficult to treat bacterial infections. Therefore, monitoring the biofilm-forming capacity of enterococci gains significance (Cui et al., 2020).

Mixed-species biofilms are the types of biofilms found predominantly in nature. Microorganisms in a mixed-species biofilm behave differently than in mono-species biofilms, especially in resistance to antimicrobials (Chen et al., 2019). Taking into account that *Salmonella* and *Enterococcus* spp. are both commonly found in the poultry intestinal microbiota, it would be unrealistic for these species to form biofilms as mono-species in poultry food processing plants. Even though research studies for the detection of *Salmonella* and *Enterococcus* spp. in poultry have been reported previously (Agarwal et al., 2011; Jahan & Holley, 2014), no studies were found on the mixed-species biofilm-forming ability of *Salmonella* and *Enterococcus* spp. Therefore, there is a need to analyze the interactions between various microorganisms during the formation of mixed-species biofilms. For this purpose, the microtiter plate method is a well-known and commonly used method for the investigation of biofilms. The plastic structure of the microtiter plate has a great advantage in examining the formation of biofilms on plastic surfaces, as plastic surfaces are very often used in the food industry (Agarwal et al., 2011; Hall-Stoodley et al., 2004).

In this study, we investigated the biofilm formation capacities of the predominant *Salmonella* and *Enterococcus* spp. such as *S. Infantis*, *S*. Kentucky, *E. faecium*, and *E. faecalis* isolated from poultry meat and slaughterhouses. The aim of this study was to experimentally reveal the synergistic and antagonistic effects of mono- and mixed-species on the formation of biofilm.

Method

Bacterial Strains and Culture Preparation

Previously isolated and well-identified *Salmonella* and *Enterococcus* strains were included in the study. Two *Salmonella* strains (*S. Infantis* HS3043 and *S*. Kentucky HS2331) were isolated from scalding tank water (Diker et al., 2020), and two *Enterococcus* strains (*E. faecalis* E54 and *E. faecium* E66) were isolated from chicken meats (Onaran et al., 2019). A single purified colony of each strain, stored in Tryptic Soy Broth (TSB, Oxoid CM0129) with 20% glycerol at −80°C, was resuscitated by adding 200 µL of stock solution into TSB and incubating at 37°C for 24 hours. All strains were subsequently subcultured once more by adding 10 µL aliquot to 10 mL of TSB to obtain a working culture. After 24 hours of incubation, serial dilutions were streaked on Tryptic Soy Agar (TSA, Merck 105458) and incubated at 37°C for 24 hours to determine the cell numbers of the stock solutions.

Biofilm Formation on 96-well Polystyrene Plates

On the day of experiment, serial dilutions were made with TSB with the bacterial strains at the late exponential phase in order to obtain 109, 107, 105, and 10^3 cfu/mL concentrations. To establish mono- and mixed-species biofilm models, the volume of bacterial strains used were as follows: (i) for mono-strains 200 µL, (ii) for dual-strains 100 µL + 100 µL each, (iii) for triple-strains 66 µL each, and (iv) for quartet-strains 50 µL each of the relevant dilutions were added to the wells of 96-well polystyrene microtiter plates. For negative control, the wells were filled with TSB only. The plates were incubated at room temperature (21 ± 1°C) for 48 hours without shaking. Each strain combination was tested in triplicate.

Quantification of Biofilm by Crystal Violet Staining

At the end of the incubation, the fluids in the plates were poured off and the wells washed three times with physiological saline solution (PSS, .9%) to remove non-adhered cells. The formed biofilms were fixed at 60°C for 15 minutes, and then the microplates were stained with 250 µL of .5% crystal violet (BDH strains, 34024) for 15 minutes at room temperature. Excess strain was poured off and the wells washed six times with PSS. The plates were air-dried and the stained biofilms were resolubilized with 250 µL of ethanol–acetone (80:20) solution (Keklikoglu et al., 2019). After 10 minutes, the optical density was measured at 590 nm (OD_{590nm}) using a microplate reader (SpectraMax i3, Molecular Devices, CA). The mono-species cultures were classified basing their optical densities as previously described by Stepanovic et al. (2003) as follows: The cutoff OD (ODc) was determined as the mean value of the negative control. $OD \leq ODc$ = non-biofilm producer, $ODc < OD \leq (2 \times ODc)$ = weak biofilm producer, $(2 \times ODc) < OD \leq (4 \times ODc)$ = moderate biofilm producer, and $OD > (4 \times ODc)$ = strong biofilm producer.

Statistical Analysis

One-way ANOVA was performed to determine the difference between bacteria and concentration groups in biofilm optical densities. The values were expressed as the mean ± standard deviation. The statistical significance level was considered as $p < .05$. All statistical analyses were performed using the IBM SPSS Statistics for Windows, Version 23.0.

Results

S. Infantis, *S.* Kentucky, *E. faecalis*, and *E. faecium* strains were examined for synergistic or antagonistic effects when co-incubated with each other at different concentrations at room temperature (21 ± 1°C) for 48 hours. OD_c at 590 nm was determined as .07 ± .01. When comparing OD_C with the mean ODs' of mono-strains independent from concentration groups, *S. Infantis* was significantly ($p < .05$) found to be the most biofilm-producing strain with $OD_{590nm} = .42 \pm .23$, and was classified as a strong biofilm producer. *E. faecium* and *S.* Kentucky were classified as weak biofilm producers with the OD_{590nm} values of .15 ± .06 and .12 ± .04, respectively ($p < .05$). However, *E. faecalis* was determined as a non-biofilm producer ($OD_{590nm} = .07 \pm .01$).

According to the dual-strain combination results, it was seen that *S. Infantis–E. faecium* ($OD_{590nm} = 1.43 \pm .15$) and *S. Infantis–E. faecalis* ($OD_{590nm} = .87 \pm .44$) combinations formed three and two times more biofilm than *S. Infantis* alone, respectively ($p < .05$). On the other hand, *S. Infantis* and *S.* Kentucky exhibited an antagonistic effect with each other when co-incubated ($OD_{590nm} = .33 \pm .17$). Interestingly, weak biofilm producers *E. faecium* and *S.* Kentucky showed a synergistic effect when inoculated together ($OD_{590nm} = .26 \pm .18$). Not surprisingly, *Enterococcus* dual-species almost never produced biofilm ($OD_{590nm} = .08 \pm .02$) (Table 1).

The synergistic and antagonistic effect results of dual-strain combinations were found parallel with triple-strain combinations. The highest biomass formation was seen in the *S. Infantis–S.* Kentucky–*E. faecium* combination ($OD_{590nm} = 1.29 \pm .40$), followed by *S. Infantis–E. faecalis–E. faecium* ($OD_{590nm} = .93 \pm .43$). Finally, when all four strains were inoculated together, .51 ± .22 was recorded at OD_{590nm} which was lower than the *S. Infantis–E. faecium* and *S. Infantis–E. faecalis* dual-strain and triple-strain combinations, but higher than all of the mono-strains.

In the study, biofilm-forming capacities of the four strains at different concentration levels (10^9, 10^7, 10^5, and 10^3 cfu/mL) were also examined. The lowest biofilm formation was seen at 10^7 cfu/mL (Figure 1). In particular, the strong biofilm producer *S. Infantis* showed a significant decrease at 10^7 cfu/mL. This decrease was also seen in triple-strain combinations of *S. Infantis–S.* Kentucky–*E. faecalis,* and *S. Infantis–E. faecalis–E. faecium,* and the quartet-strain combination at 10^7 cfu/mL ($p < .05$). However, no significance was observed within concentration groups of 10^9, 10^5, and 10^3 cfu/mL in general ($p > .05$).

Discussion, Conclusion, and Recommendations

There have been numerous studies displaying the biofilm formation of *Salmonella* and enterococci as single strains (Agarwal et al., 2011; Da Silva Fernandes et al., 2017; Jahan & Holley, 2014; Stepanović et al., 2003). It can be concluded from these

Table 1

Biofilm Formation by Salmonella Infantis, Salmonella Kentucky, Enterococcus faecalis, and Enterococcus faecium Mono- and Mixed-Species at Room Temperature (21 ± 1°C) for 48 Hours, Independent From Concentration Groups

Bacteria	Mean ± SD	Minimum–Maximum
Negative control	.07 ± .01	.06–.09
SI	.42 ± .23	.13–.79
SK	.12 ± .04	.07–.19
EFe	.07 ± .01	.06–.08
EFa	.15 ± .06	.09–.25
SI+SK	.33 ± .17	.10–.59
EFe+EFa	.08 ± .02	.06–.12
SI+EFa	1.43 ± .51	.74–2.43
SI+EFe	.87 ± .44	.25–1.61
SK+EFa	.26 ± .18	.10–.64
SK+EFe	.15 ± .05	.09–.24
SI+SK+EFa	1.29 ± .40	.86–2.06
SI+SK+EFe	.76 ± .32	.31–1.36
SI+EFa+EFe	.93 ± .43	.40–1.69
SK+EFa+EFe	.25 ± .13	.10–.54
SI+SK+EFa+EFe	.51 ± .22	.19–.87

Note: SD = Standard deviation; SI = *Salmonella Infantis*; SK = *Salmonella Kentucky*; EFe = *Enterococcus faecalis*; EFa = *Enterococcus faecium*.

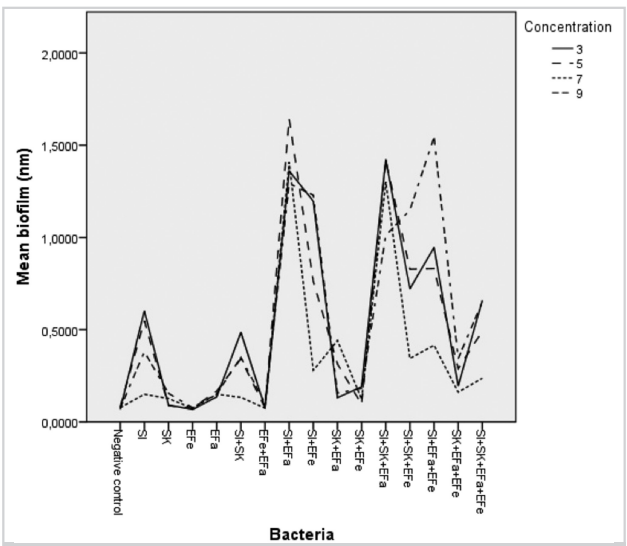

Figure 1

Mono- and Mixed-Species Mean Biofilm Formations in Different Concentrations after 48 Hours Incubation at Room Temperature (21 ± 1°C).

previous studies that every strain and/or serotype is affected by some factors such as nutrients, pH, oxygen, salt, etc., and biofilm-forming ability differs among serotypes and growth kinetic behaviors. According to our results, *S. Infantis* was determined as a strong biofilm producer. This result is in agreement with the thesis study of Açıkalın (2017), where *S. Infantis* strains ($n = 100$) were defined as strong biofilm producers after incubation at 20°C for 48 hours. On the contrary, *S. Kentucky* ($n = 5$) was also found to be a strong biofilm producer in the same study. In another study, *S. Kentucky* strain was again determined as a strong biofilm producer (Agarwal et al., 2011). The reason for the differences can be explained. As mentioned above, each strain is distinct and has different biofilm production kinetics. The same situation is valid for *E. faecalis* and *E. faecium*. Jahan and Holley, (2014) displayed the biofilm-forming capacities of *E. faecalis*, *E. faecium*, and *E. gallinarum,* and observed that the biofilm production rate of each strain varies at different temperatures. Parallel with our study, they reported *E. faecium* as a weak biofilm producer.

Mixed-species results showed that *S. Infantis* and *Enterococcus* have a positive impact on biofilm formation when co-inoculated. However, when *S. Infantis* was mixed with *S. Kentucky* the OD_{590nm} was significantly lower than *S. Infantis* alone. It was clearly observed that *S. Infantis* alone produced much more biomass compared to other strains. Therefore, it is thought that the capacity of *S. Infantis* to produce biofilms is due to the lack of a competitive environment when it is with the weak producers. On the other hand, in some cases, one strain can attach to the surface much more easily and intensely, and does not allow other strains to adhere. In this situation, the bacteria which cannot attach well to the surface no longer survive in multi-species environments, even if it is a strong or moderate biofilm-producing strain. For instance, in the study conducted by Chen et al. (2015), *Salmonella* and *E. coli* O157:H7 were inoculated together and the biofilm they formed was examined under a confocal laser microscope. Although both the species were strong biofilm producers, when they were inoculated together, due to the fast and tight attachment of *Salmonella* to the surface, *E. coli* O157:H7 could not adhere and therefore could not produce biofilm. The antagonist effect in our study can be attributed to this reason.

There are very limited studies on biofilm production in environments containing more than one strain of the same species. Kostaki et al. (2012) compared the amount of biofilm formed by three serotypes of *Listeria monocytogenes* inoculated together with those produced alone, and found that each strain did not participate equally in biofilm formation in the mixed-strain community. Also, Da Silva Fernandes et al. (2017) reported that in a multi-species biofilm, *E. faecalis* and *E. faecium* cell counts were increased while *Bacillus cereus* decreased. In this current study, it was observed that the triple-strain combinations with *S. Infantis* were significantly higher than those produced by *S. Infantis* alone. However, when the four strains were together, the increase was not significant ($p > .05$). Since it is known that biofilm formation by a bacterium can be stimulated or inhibited by the presence of other bacteria, the interaction between these two species can be more clearly demonstrated using more advanced imaging techniques (Gkana et al., 2016).

There is limited knowledge about the effect of bacterial level on biofilm formation. This study also aimed to reveal the biofilm-producing capacity of selected bacteria at different concentrations. When the results are examined, there was a significant decrease in the biomass of the strong biofilm producer *S. Infantis* at the level of 10^7 cfu/mL. This decrease was also observed in triple and quartet combinations of *S. Infantis*. In addition, regardless of the type of bacteria, the lowest biomass was found again at 10^7 cfu/mL. On the other hand, it was seen that the amount of biofilm of *S. Infantis* increased at low concentrations. This relatively increased amount of biofilm at low concentrations may be associated with a less competitive environment for resources such as nutrients and oxygen in the microenvironment, in the presence of low levels of bacteria. However, further studies are needed to reveal more clearly the effect of bacterial level on biofilm formation.

Biofilm formation is an important strategy for bacteria to combat with undesirable conditions. Although each bacterial strain has its own unique capacity to form biofilm, it is crucial to understand their interactions when they settle in the same biofilm environment, as they are commonly encountered as multi-species biofilms in the food industry. Because *Salmonella* and enterococci are generally found in the poultry intestinal microbiota together, it is inevitable that a biofilm containing one of these species in poultry slaughterhouses or meat production facilities will also contain the other one. Besides, the transfer of virulence and resistance genes between species is much easier in such multi-species biofilm environments. Since all co-resident microorganisms are able to compete, cooperate, and interact with each other, the findings of the study could help in future studies targeted at combating biofilms in food processing environments.

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