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**Research Article**

# DETERMINATION OF *in vitro* BIOFILM FORMATION ABILITIES OF FOOD BORNE *Salmonella enterica* ISOLATES

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**Abstract:** Salmonellosis caused by non-typhodial *Salmonella enterica* serotypes is one of the most important food-borne diseases worldwide and biofilm structure formed by these pathogens provide a reservoir for food contamination and a source for infections. This study was performed in order to determine biofilm formation abilities of food borne *Salmonella* isolates on polystyrene and on air liquid interphase and their colony morphologies when grown on Congo Red Agar plates. 32 food-borne *Salmonella* strains isolated from retail chicken carcasses in Edirne province of Turkey and belonging to the Infantis, Enteritidis, Kentucky and Telaviv serotypes were used. The microtiter plate technique was used to determine biofilm formation abilities of the isolates on polystyrene surfaces by measuring the optical density (OD) values of the stained bacterial biofilms. The results showed that the strongest biofilm formation capacities of the isolates were observed at 22°C for 3 days of incubation. Although all isolates formed pellicle on the liquid-air interface at 22°C, only 13% of the isolates belonging to the Infantis, Kentucky and Enteritidis serovars formed pellicle at liquid-air interface at 37°C. Three different colony morphotypes (saw; smooth and white, bdar; brown, dry and rough, rdar; red, dry and rough) were determined on Congo Red Agar among the isolates. High biofilm formation abilities of the tested *Salmonella* isolates can lead to widespread of virulence and resistance properties, especially to medically important antibiotics such as ciprofloxacin, via food chain. This situation constitutes an important concern for public health.

***Key words:*** *Salmonella*, biofilm, food-borne pathogen, microbial food safety.

**Özet:** *Salmonella enterica* serotiplerinin neden olduğu salmonelloz vakaları dünya çapında en önemli gıda kökenli hastalıkların başında gelmektedir. Bu patojenlerin oluşturduğu biyofilm yapısı hem gıda kontaminasyonlarına neden olmakta hem de enfeksiyonlar için kaynak oluşturmaktadır. Bu çalışmada gıda kökenli *Salmonella enterica* izolatlarının polistirende ve hava-sıvı ara fazında biyofilm oluşturma yetenekleri ve ayrıca Kongo kırmızısı agar plakalarında koloni morfolojilerinin belirlenmesi amaçlanmıştır. Çalışmada Edirne ilinde satışa sunulan tavuk karkaslarından izole edilmiş 32 adet *Salmonella* izolatı kullanılmıştır. İzolatların plastik yüzeylerdeki biyofilm oluşturma yeteneklerinin belirlenmesinde boyanmış bakteriyel biyofilmlerin optik yoğunluklarının ölçülmesi esasına dayanan mikroplaka tekniği kullanılmıştır. Polistren plakalardaki biyofilm oluşturma sonuçlarına göre, izolatların en güçlü biyofilm oluşturma kapasitesi 22°C'de 3 günde gözlemlenmiştir. Bütün izolatlar 22°C'de sıvı-hava ara fazında pelikül oluştururken izolatların sadece % 13'ü (Infantis, Kentucky ve Enteritidis serovarlarını içeren) 37°C'de sıvı-hava ara fazında pelikül oluşturmaktadır. Kongo kırmızısı agarda üç farklı koloni morfolojisi (saw; pürüzsüz ve beyaz, bdar; kahverengi, kuru ve pürüzlü, rdar; kırmızı, kuru ve pürüzlü) belirlenmiştir. *Salmonella* izolatlarının yüksek biyofilm oluşturma kapasiteleri, virülens ve özellikle tıbbi olarak önemli antibiyotikleri içeren direnç faktörlerinin gıda zinciri yolu ile geniş çaplı yayılımına neden olabilir. Bu durum halk sağlığı açısından büyük bir endişe kaynağı oluşturmaktadır.

## Introduction

Salmonellosis caused by non-typhodial *Salmonella enterica* serotypes is one of the most important food-borne diseases worldwide and an increasing number of human Salmonellosis cases have been linked to consumption of contaminated food (Panisello *et al.* 2000). Poultry and poultry associated products in particular are essential reservoirs for food-borne pathogens and these products have been identified as significant transmission vehicle for Salmonellosis by allowing *Salmonella* to be easily transmitted to humans from these products (Hoelzer *et al.* 2011, Antunes *et al.* 2016). It is also known that many pathogenic bacteria including *Salmonella* can attach to and grow on food and food contact surfaces to form biofilms in food processing industry which cause major economic and public health concerns and this biofilm formation ability can be the main reason of wide distribution of *Salmonella* isolates (Vestby 2009b). Biofilm formation depends on some characteristics of the bacteria and environmental factors such as pH, temperature and nutrient components (Shia & Zhu 2009).

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Bacterial biofilms are highly resistant to environmental stress, antibiotics, and disinfectants and thus are difficult to sanitate (Hoiby *et al.* 2010, Steenackers *et al.* 2012). Biofilms constitute a source for clinical infections and pathogenic microorganisms in biofilms provide a reservoir for food contaminations (Costerton *et al.* 1999, Srey *et al.* 2013).

In the present study, biofilm formation abilities of food borne *Salmonella* isolates on an abiotic surface (polystyrene plate) were investigated. The tests were performed at three different incubation temperatures and times to determine how these variables affected biofilm formation by the isolates. The pellicle formation on air liquid interphase was also tested as an indication of biofilm formation. In addition, colony morphologies of the isolates on Congo Red Agar plates were evaluated to reveal the components of biofilm produced considering morphotypes on the agar plates.

## Materials and Methods

### Bacterial strains

*Salmonella* strains formerly isolated from retail chicken carcasses in Edirne province of Turkey were used (see Aksoy and Şen 2015). The strains were determined to belong to the Infantis (n=26), Enteritidis (n=4), Kentucky (n=1) and Telaviv (n=1) serotypes. The original codes assigned to the serotypes (from A1 to A32) were used throughout the text when it was necessary to refer to the serotypes individually.

### Biofilm formation on polystyrene

96-well polystyrene microplates (Grenier Bio-One) were used for quantification of biofilm production based on the previously described method with some modifications (Stepanović *et al.* 2004). The wells were filled with 230 µL of LB$^{wo}$/NaCl broth. Liquid bacterial cultures (18 h old) were diluted in LB$^{wo}$/NaCl broth to OD570 = 0.2 and 20 µL of these cultures were transferred to each well (three parallels of each strain). The negative control wells contained broth only. Microplates were incubated statically for one (24 h), two (48 h) and three days (72 h) at 5°C, 22°C and 37°C. The incubation temperatures were selected to represent routine storage condition of foods, room temperature and optimum growth condition of *Salmonella*, respectively. After incubation, the contents of the wells were emptied, wells were washed three times with 300 µL sterile distilled water and then 250 µL of methanol (≥ 99.9 %) was added per well. After incubation for 15 min in room temperature, microplates were emptied and air dried. Then wells were stained with Cyristal violet (0.1%) used for Gram staining (Merck) for 5 min. Excess stain was rinsed off by washing the microplates with running tap water and the microplates were air dried. The dye bound to adherent cells was dissolved using 250 µL of 33 % (v/v) glacial acetic acid in each well. The optical density was measured at 570 nm using a Multiskan EX reader (Bio-Rad). Based on the OD results, strains were classified into four categories as no biofilm producers, weak, moderate or strong biofilm producers as previously described (Stepanović *et al.* 2000). The cut-off optical density (ODc) is defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: OD ≤ ODc = no biofilm producer, ODc < O.D. ≤ (2 x ODc) = weak biofilm producer, (2 x ODc) < OD ≤ (4 x ODc) = moderate biofilm producer and (4 x ODc) < OD = strong biofilm producer.

### Pellicle formation at liquid-air interface

The pellicle formation at the liquid-air interface was tested by inoculation of 0.5 mL of bacterial cultures (18h) in 4.5 mL LB $^{wo}$/NaCl for incubation at 5°C, 22°C and 37°C for eight days in static conditions. The strains were visually controlled every day for presence of pellicle formation (Solano *et al.* 2002). *S.* Typhimurium ATCC 14028 was used as the positive control.

### Colony morphology on Congo Red (CR) Agar plates

The method described by Turki *et al.* (2012) was used with some modifications for determination of colony morphology. Bacterial cultures (18 h old) were plated on LB $^{wo}$/NaCl agar supplemented with Congo Red (8 µg/mL). After incubation at 5°C, 22°C and 37°C for eight days, the colony morphology was determined based on the classification as saw (smooth and white), bdar (brown, dry and rough), and rdar (red, dry and rough). *S.* Typhimurium ATCC 14028 was used as the positive control for rdar morphology.

### Statistical Analyses

Kruskal-Wallis test was used to calculate the significance of the differences between the biofilm formation abilities of the isolates on microtiter plates at different incubation times and temperatures ($p<0.05$). The differences between average biofilm formations on polystyrene by the bdar and rdar morphotypes were analyzed by the Student's t-test ($p<0.05$). Spearman's correlation test was used to determine the association between biofilm formation of the isolates on microtiter plates and their pellicle formation abilities. Analyses were performed with Graphpad PRISM software (Intuitive Software for Science, San Diego, CA).

## Results and Discussion

In the present study, biofilm formation abilities of food borne *Salmonella* isolates belonging to four different serovars (Infantis, Enteritidis, Kentucky and Telaviv) were determined on polystyrene microtiter plates at different temperatures representing routine food storage conditions, room temperature and optimum growth condition of *Salmonella*, respectively. The effect of incubation temperature and times on biofilm formation ability of the isolates on microtiter plates is shown in Fig. 1.

The hygienic status of food contact and processing surfaces is very important and determined by the ability of cells to adhere to surfaces (van Houdt & Michiels, 2010). Polystyrene is a hydrophobic material and widely used in production of food contact, food packing and food service materials (Genualdi *et al.* 2014).

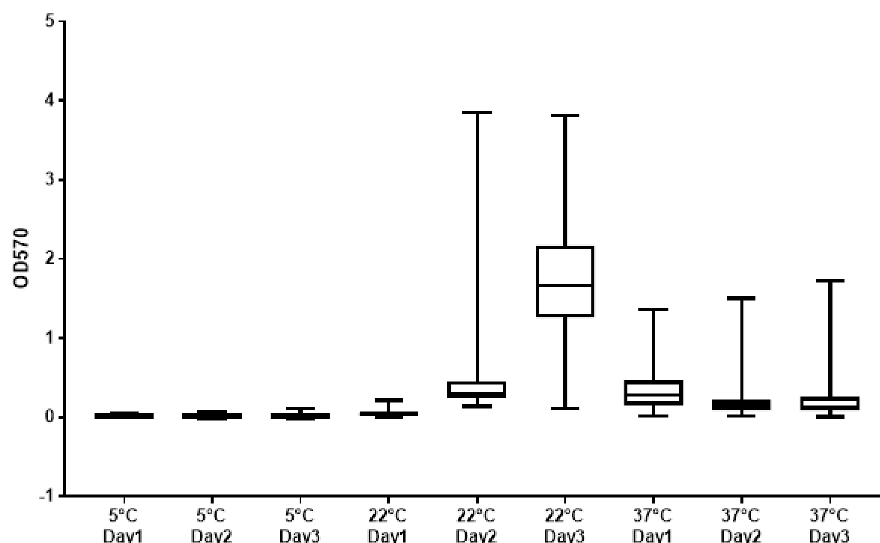


**Fig. 1.** The OD570 values of *Salmonella* isolates on microtiter plates at different incubation temperatures (5°C, 22°C and 37°C) and incubation times (1, 2 and 3 days). The OD value at 570 nm was used as the indication of level of biofilm formation.

Researchears commonly use microtiter plates as an *in vitro* mimic of the plastic surfaces in poultry production and daily use in houses (Díez-García *et al.* 2012, Piras *et al.* 2015, Borges *et al.* 2018). It has been previously showed that *Salmonella* spp. are capable of producing more biofilm on hydrophobic surfaces than hydrophilic surfaces (suchs as stainless steel, glass) (Sinde & Carballo 2000, Donlan 2002, Cunliffe *et al.* 1999). In a similar manner, biofilm formation was determined for all of the *Salmonella* isolates at 22°C (day1 and day2). When the average biofilm formation of the isolates on microtiter plates were compared, the results showed that there was no significant difference between different incubation days at 5°C while the differences at 22°C were significant ($p<0.05$). When the incubation temperature was 37°C, the difference between day1 and day2 was significant ($p<0.05$) but between day2 and day3 was not.

When the different incubation temperatures were compared based on a daily manner, the results revealed significant differences for all pairs for all days except 5°C and 22°C for day1. Biofilm forming abilities of bacteria depend on multiple factors one of which is the bacterial cell surface. For instance, in *S.* Typhimurium, expression of thin aggregative fimbriae was reported to increase at lower temperatures (20 and 28°C) (Römling *et al.* 1998). Increase in adherence to a surface at a low temperature may lead to persistence and survival in food-processing environments (Van Houdt and Michiels, 2010). It has been previously demonstrated that lower incubation temperature induced the biofim formation capacities of *Salmonella* serovars (Stepanović *et al.* 2003, Karaca *et al.* 2013, Milanov *et al.* 2017).

Accordingly, our results showed that the strongest biofilm formation capacities of the isolates were observed at 22°C for 3 days (Fig. 1.). At 37°C for 3 days, 22% of the isolates (Enteritidis and Infantis serovars) were determined as strong biofilm producers, 22% (Infantis and Kentucky serovars) were determined as moderate biofilm producers and 56% (Infantis and Telaviv serovars) were determined as weak biofilm producers. At 22°C (room temperature), 98% of the isolates (Enteridis, Infantis, Kentucky and Telaviv serovars) were determined as strong biofilm producers whereas only 2% (Enteritidis serovar) was determined as weak biofilm producers. At 5°C, 44% of the isolates (Enteritidis and Infantis serovars) were determined as weak biofilm producers and 56% (Infantis, Kentuck and Telaviv serovars) produced no biofilm. The lowest OD570 value (0.001) was obtained in one of the Infantis isolates (A27) with the incubation at 5°C for 1 day and the highest OD570 value (3,820) was obtained in the Telaviv isolate (A22) with the incubation at 22°C for 3 days. There are a few studies regarding the effects of incubation period and temperature on biofilm formation of *Salmonella* spp. strains. In a former study, Stepanović *et al.* (2003) studied with the serovars Enteritidis and Typhimurium, and showed that, although there was a significant difference in biofilm formed at 22°C incubation temperature after 24 and 48 h incubations, there was no significant difference when the temperature was set at 30°C/37°C for the same incubation periods. More recently, Vestby *et al.* (2009b) tested the effect of incubation period on biofilm formation in microtiter plates using the serovar Typhimurium, Agona, Montevideo, Senftenberg strains and found that only the serovar Sefteenberg strains exhibited increased biofilm forming capacities for 2 and 4 days of incubation. In the present study, the effects of prolonged incubation period and different incubation temperatures were tested on biofilm formation in microtiter plates based on a serovar manner. Serovar Infantis, Enteritidis, Kentucky and Telaviv isolates were used, of which Infantis, Enteritidis and Kentucky serovars are among the most common isolated serovars in Turkey. Interestingly, Telaviv has also been commonly isolated from food samples in Turkey although it is among the rare serovars throughout

the world (Ozdemir & Acar 2014, Erol 1999, Durul *et al.* 2015). The results showed that both the prolonged incubation period and the temperature effected the biofilm formation capacities depending on the serovars. Serovar Infantis strains were the only serovars which showed significant increase in OD570 values from day one to day three for all incubation temperature conditions (5°C, 22°C and 37°C). Serovar Telaviv and Kentucky strains showed significant increase only at 22°C and serovar Enteritidis strains showed significant increase only at 5°C. It was suggested that at 22°C, in the starionary phase of growth, utilization of nitrogen and phosphate induced the agfD promotor and contributed the multi-cellular state, but optimum growth condition (37°C) caused faster changes in oxygen tension and pH and this situation had a negative effect on multicellular status of *S.* Typhimurium (Gerstel and Romling, 2001, Stepanović *et al.* 2003). The results obtained in the present study revealed the importance of evaluation of all these factors on a serovar basis. These results reveal the necessity of further and more detailed studies on biofilm formation capacities of different serovars and isolates.

Three different colony morphotypes (saw, bdar, and rdar) of the isolates were determined on CR Agar. rdar which is caused by the coexpression of curli fimbriae and cellulose is a typical colony morphology that was observed between biofilm producer isolates. bdar and saw morphotypes were determined with deletion mutation in csg genes required for curli fimbriae synthesis or in both csg and bcs genes coding for cellulose synthesis, respectively. The production of cellulose and curli fimbriae is important in biofilm formation and its persistence on various surfaces (Römling, 2000, Cookson *et al.* 2002, Solano *et al.* 2002). When incubated at 37°C, 94% of the isolates exhibited bdar, 3% exhibited saw morphotype and one isolate belonging to the Infantis serovar presented a morphotype that has not been described previously. When the incubation temperature was 22°C, 81% and 19% of the isolates presented bdar and rdar morphotypes, respectively. On the other hand, isolates did not form biofilm on CR agar at low temperature (5°C). bdar and rdar morphotypes were good biofilm producers and both morphotype were commonly detected in *Salmonella* strains but studies showed that rdar strain was more tolerant to long-term desiccation and nutrient depletion in biofilm when compared to the bdar strain. (Vestby *et al.* 2009a). Average biofilm formations on polystyrene by the bdar and rdar morphotypes at 22°C were determined as 1.483±0.098 and 2.438±0.246, respectively and the difference between the two OD values was statistically significant ($p<0.05$) (Fig. 2).

When the different morphotypes were evaluated in terms of the serovars, the bdar morphotype was found to be exhibited by 96% of the Infantis serovars while the Enteritidis, Kentucky and Telaviv serovars isolates exhibited the rdar morphotype. Also, a similar serovar-morphotype relation was determined in a former study (Karaca *et al.* 2013).

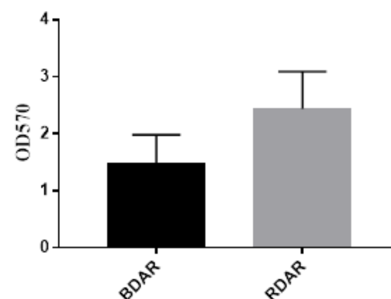


**Fig. 2.** Average biofilm formation on polystyrene of bdar and rdar strains.

Although all isolates formed pellicle at liquid-air interface at 22°C, only 13% of the isolates belonging to the Infantis (A3, A16), Kentucky (A10) and Enteritidis (A13) serovars formed pellicle at liquid-air interface at 37°C. No pellicle formation was detected at 5°C. No correlation was determined between biofilm formation of the isolates on microtiter plates and their pellicle formation abilities ($r=0.1545$, $p>0.05$). rdar and bdar morphotyped isolates formed pellicle with rigid and fragile structures, respectively.

In a recent study, antibiotic resistance profiles of the isolates used in the present study were determined (Aksoy & Şen 2015). 81.3% of the isolates exhibited multiple antibiotic resistance and *S.* Kentucky (A10) and *S.* Enteritidis (A32) isolates were resistant to ciprofloxacin. Ciprofloxacin resistance is important because it is a common antibiotic choice for invasive salmonellosis in humans (Velhner *et al.* 2014). 67.75% of the isolates were found to have high pathogenicity potential in *Caenorhabditis elegans* nematode model system (Aksoy & Şen 2015).

In a biofilm, avirulent and sensitive strains can become virulence and resistant via acquisition of virulence and resistance genes. It is also known that the rate of fluoroquinolone resistance of *Salmonella* strains was very low in Turkey (Albayrak *et al.* 2004, Ercis *et al.* 2006). However, fluoroquinolone resistance has recently become a common problem (Lin *et al.* 2015). High biofilm formation abilities of the present *Salmonella* strains can lead to widespread of virulence and resistance properties, especially to medically important antibiotics such as ciprofloxacin, via food chain. In addition, this situation constitutes an important public health concern. It is also known that biofilm formation abilities of *Salmonella* isolates are facilitated by long term persistence in food production environments (Vestby *et al.* 2009b). The present results suggested that almost all *Salmonella* isolates tested produced biofilm at room temperature. As a result, persistency of these isolates on abiotic surface increases which in turn increases cross contamination risk of the isolates. This can also be an explanation of widely distribution of *Salmonella* isolates in food. It is also an important public concern because poor sanitation of surfaces that comes in contact with food causes food borne outbreaks. This situation revealed the

importance of microbial food safety and sanitation in industrial processes. Further investigations are required to investigate sanitation methods and effects of disinfectants on biofilms. An efficient and environmentally friendly control strategy is desired to better meet food safety and processing necessities, keeping in mind the fact that the resistance of biofilms to conventional disinfection processes is increased. In addition, interrupting the quorum sensing abilities of biofilm forming bacteria by natural biological agents may also be an affective option to solve biofilm related problems.

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