Biofilm Production Potential of *Salmonella* Enteritidis and *Salmonella* Infantis Serovars Isolated from Broiler Flocks

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ABSTRACT

In recent years, it has been emphasized that biofilms formed by *Salmonella*, one of the worldwide zoonotic pathogens, is a factor that supports resistance to disinfectants and antibiotics, and that some serotypes with multiple antibiotic resistance spread in this way. This study aimed to examine the biofilm structures formed by *Salmonella* serovars (*S*. Enteritidis and *S*. Infantis), from poultry litter isolates, under different environmental conditions and to determine the optimum conditions. The biofilm formation potential of the strains at different temperatures (20˚C and 37˚C) during 24, 48 and 72 hours of incubation was examined. It was determined that all strains formed biofilms at different degrees (weak, medium, and strong). For strong biofilm formation, 48 and 72 hours of incubation at 20˚C or 72 hours of incubation at 20˚C and 37˚C were considered suitable for the *Salmonella* serovars used in this research. The findings of this study indicate that determining the biofilm-forming potential of *Salmonella* Enteritidis and *Salmonella* Infantis serovars will make a significant contribution to our understanding of the resistance mechanisms of these pathogens in the industrial and medical sectors aid the development of effective strategies to prevent their spread.

Key words: Biofilm formation; Broiler flocks; *Salmonella*; *S*. Infantis; *S*. Enteritidis.

INTRODUCTION

Salmonella is among the most significant zoonotic pathogens that can be found in the intestines of many animal species, including humans, wild birds, domestic animals and rodents. Furhtermore, These bacteria can result in both acute and chronic illnesses in poultry flocks, and can be spread from infected poultry to humans (1). *Salmonella* appears to rank second amongst foodborne gastrointestinal diseases with reported salmonella cases worldwide (2). Studies show that salmonellosis detected in humans is caused by contaminated materials such as milk and dairy products, seafood, poultry, eggs and meat products (3,4). In addition to *Salmonella* Typhimurium and *Salmonella* Enteritidis serovars, which are known to be among the most frequently isolated serotypes in human salmonellosis cases, it has been reported that the severity of *Salmonella* Infantis in salmonellosis cases has rapidly increased in recent years (2).

Today, we see the negative consequences of the uncontrolled use of antibiotics, ranging from food to animal and human health, with the spread of antibiotic-resistant strains. This reveals the seriousness of the situation with the emergence of multiple drug resistance (MDR) strains isolated from human, animal and poultry samples in many parts of the world. The emergence of MDR can make treatment difficult and cause infections and health problems (5). Various studies have emphasized that antibiotic resistance detected in *Salmonella* serovars should be focused on biofilm-forming properties that make treatment and eradication challenging (6-10).

The term biofilm is defined as "communities of micro-

organisms that irreversibly adhere to inanimate or living surfaces and behave like multicellular organisms in the extracellular polymeric matrix they produce" (11). Biofilm matrix is produced by biofilm-forming bacteria and provides mechanical and chemical protection against the external environment. It is known that *Salmonella* is an ideal biofilm producer in humid environments where for example in poultryfarming, in ventilators, coolers, drinkers, water tanks and sites with air contact (11,12). The genetic expression of *Salmonella* is also affected by environmental conditions. Studies have reported that other environmental factors such as nutriental status of the hosts, pH, temperature, osmolarity, iron and oxygen all affect biofilm development (6).

Studies have evaluated that *Salmonella* can maintain their viability for a long period of time and their infections can spread rapidly in a short time, with their ability to form cross-contaminations and biofilms (4). In the literature, it is considered that the identification of *Salmonella* serovars, antibiotic resistance profiles and determination of biofilm formation parameters that make eradication difficult, are necessary for epidemiological surveillance and disease evaluation (5).

The aim and approach in finding effective solutions to *Salmonella* infections, which pose an important clinical and industrial problem, is to determine the main factors that are effective during biofilm production. Considering these principles, in this study; it was aimed to determine the biofilm formation potential of *S*. Infantis and *S*. Enteritidis serovars in different environmental conditions.

MATERIAL AND METHODS

Bacterial Strains

In this research, *S*. Enteritidis (n:3; Lab.Code:N12, N13, N24) and *S*. Infantis (n:3; Lab. Code:N9, N10, N11) serovars isolated from poultry litter from commercial broiler facilities belonging to the bacterial culture collection of Department of Microbiology, Faculty of Veterinary Medicine, Aydin Adnan Menderes University, Turkiye were used. The serovars are known to have MDR.

Biofilm Assay

To assess the biofilm-forming potential of *Salmonella* strains, the method applied by Stepanović *et al*. (2004) was modified and used (13). Briefly, 180 µl of sterile Tryptic Soy Broth (TSB) (Merck, Darmstadt, Germany) medium prepared by adding 0.25% glucose was taken and distributed into 96-well flat-bottom microplates (Greiner bio-one, Hamburg, Germany). 20 µl of *Salmonella* bacterial culture, previously adjusted to a McFarland 0.5 standard, was added into the microplates. The prepared microplates were incubated at 20˚C and 37˚C for 24, 48 and 72 hours to determine biofilm formation. At the end of the incubation, the microplates were emptied, washed with sterile phosphate buffer, and methanol was added. After 20 minutes, the microplates were emptied, inverted and dried at room temperature. Then, 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) solution was added to the wells and left for 15 minutes. At the end of the period, after the microplates were washed and dried, 95% ethanol was added to the wells.

Spectrophotometric microplate measurement method was used in the evaluation. The optical density was measured at 595 nm (OD_{595}) using a Multiskan EX reader (Labsystems, Helsinki, Finland). Cut-off OD (ODc) was determined as the average value of the negative control over the medium that did not contain bacteria and was used as a negative control in the study. All calculations from Stepanović *et al*. (2000) was implemented as recommended (14). All tests were performed in triplicate. The arithmetic means (OD_{ort}) of the obtained OD values were used to compare biofilm production times and to explain the effect of environmental factors and temperature on the biofilm. According to the optical density values, biofilm formation was interpreted as negative, weak, medium or strong.

RESULTS

Quantitative detection of biofilm formation was performed by the microplate method. Bacteria-free medium was used as a negative control. The cut-off ODc value calculated from the bacteria-free medium before incubation was calculated as 0.091. It was determined that all *Salmonella* strains produced strong biofilms after 48 hours of incubation at 20˚C, and at the end of the 72-hour incubation period, the strains continued to produce strong biofilms. It was noted that *S*. Enteritidis N12, N13, N24 and *S*. Infantis N9 strains produced moderate biofilms as a result of 24-hour incubation at 20˚C, while *S*. Infantis N10 and N11 strains produced weak biofilms. It was determined that all strains produced moderate biofilms after 24 and 48 hours of incubation at

Table 1. Degree of biofilm formation by *Salmonella* serovars.

Incubation time:	24hr		48hr		72hr	
Incubation temperature:	**20°C**	**37°C**	**20°C**	**37°C**	**20°C**	**37°C**
S. Enteritidis N12	medium	medium	strong	medium	strong	strong
S. Enteritidis N13	medium	medium	strong	medium	strong	strong
S. Enteritidis N24	medium	medium	strong	medium	strong	strong
S. Infantis N9	medium	medium	strong	medium	strong	strong
S. Infantis N10	weak	medium	strong	medium	strong	strong
S. Infantis N11	weak	medium	strong	medium	strong	strong
Negative control	–	–	–	–	–	–

Table 2. Incubation temperature and times on biofilm formation ability of serovars

Incubation Time/ Incubation Temperature						
ODort values of serovars	**24hr/20°C**	**24hr/37°C**	**48hr/20°C**	**48hr/37°C**	**72hr/20°C**	**72hr/37°C**
S. Enteritidis N12	0.3088	0.3428	0.4026	0.2825	1.4571	0.7479
S. Enteritidis N13	0.2151	0.3285	0.6414	0.3002	0.6842	0.6248
S. Enteritidis N24	0.2309	0.3564	0.7853	0.3158	1.3604	0.5695
ODort values of N12, N13, and N24	0.2516	0.3426	0.6098	0.2995	1.1672	0.6474
S. Infantis N9	0.2017	0.3322	1.6206	0.2967	1.0711	0.5082
S. Infantis N10	0.1424	0.3561	0.8357	0.3300	1.0117	0.5692
S. Infantis N11	0.1262	0.3511	0.5483	0.2943	1.0592	0.6152
ODort values of N9, N10, and N11	0.1568	0.3465	1.0015	0.3070	1.0473	0.5642
OD_{ort} values of negative control	0.0910	0.0910	0.0910	0.0910	0.0910	0.0910

37°C and strong biofilms at the end of 72 hours of incubation (Table 1).

The optical density (OD) values for S. Enteritidis strains N12, N13, and N24 were assessed at 24, 48, and 72 hours of incubation at both 20°C and 37°C. At 20°C, the OD values after 72 hours indicated peak biofilm formation, with values of 1.4571, 0.6842, and 1.3604, respectively. Similarly, for *S.* Infantis strains N10 and N11, OD values were measured at 24, 48, and 72 hours at both temperatures. After 72 hours at 20°C, the OD values were 1.0117 and 1.0592, respectively. The *S.* Infantis N9 strain demonstrated the highest biofilm formation with an OD value of 1.6206 after 48 hours at 20°C. The impact of incubation temperature and duration on biofilm formation by these isolates is illustrated in Figure 1 and summarized in Table 2.

After 24 hours of incubation at 20°C incubation temperature, OD_{ort} for *S.* Enteritidis N12, N13, N24 was 0.2516; OD_{ort} was calculated as 0.1568 for *S.* Infantis N9, N10, N11. After 48 hours of incubation, OD_{ort} for *S.* Enteritidis N12, N13, N24 was 0.6098; OD_{ort} for *S.* Infantis N9, N10, N11 was determined as 1.0015. After 72 hours of incubation, OD_{ort} for *S.* Enteritidis N12, N13, N24 was 1.1672; OD_{ort} for *S.* Infantis N9, N10, N11 was determined as 1.0473, (Table 2).

Following a 24-hour incubation period at 37°C, OD_{ort} for *S.* Enteritidis N12, N13, N24 was 0.3426; OD_{ort} was calculated as 0.3465 for *S.* Infantis N9, N10, N11. After 48 hours of incubation, OD_{ort} for *S.* Enteritidis N12, N13, N24 was 0.2995; OD_{ort} for *S.* Infantis N9, N10, N11 was determined as 0.3070. After 72 hours of incubation, OD_{ort} for *S.* Enteritidis N12, N13, N24 was 0.6474; OD_{ort} for *S.* Infantis N9, N10, N11 was determined as 0.5642 (Table 2).

DISCUSSION

In recent years, resistance to antibiotics and the spread of resistant bacteria have emerged as an important global public health problem (10, 15). In parallel, it is clear that

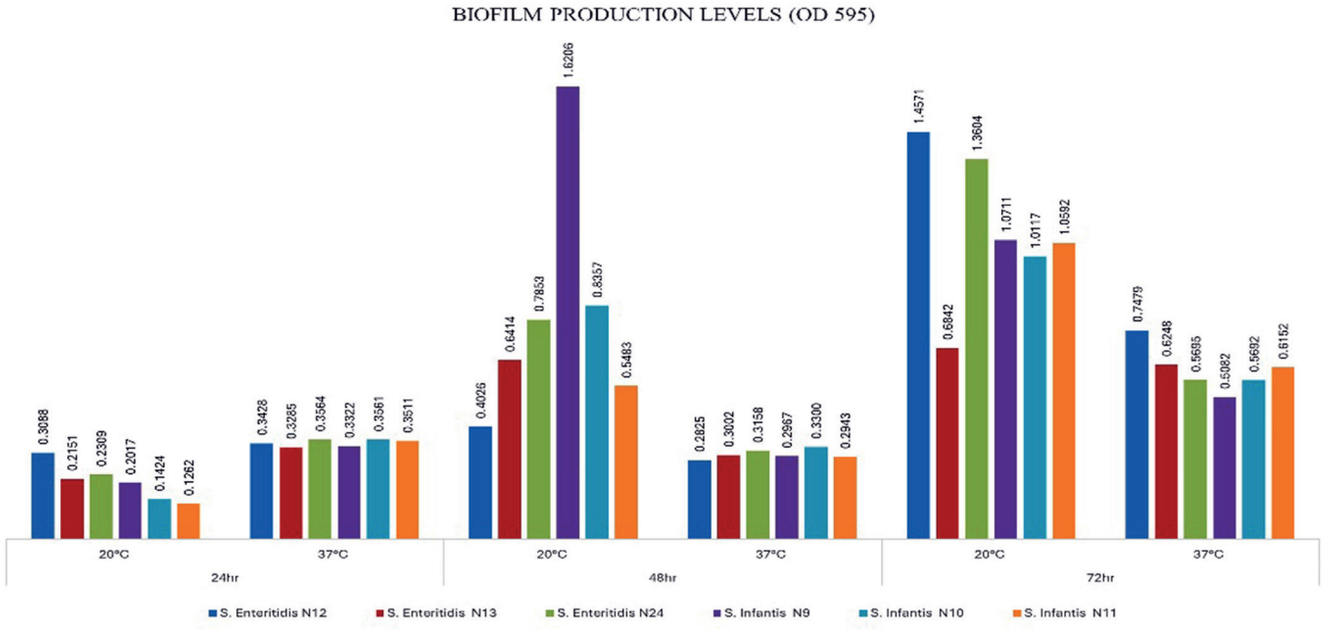

Figure 1. The effect of incubation temperature and times on biofilm formation ability of serovars. OD_{ort} values of Salmonella serovars. OD_{ort} values of negative control is 0.0910.

increasing antibiotic resistance makes it difficult to control *Salmonella* infections. It is reported that *S.* Infantis colonizes the digestive system of animals in broiler farms and spreads around with feces, contaminating the environment and the surrounding chickens, and its potential to develop antimicrobial resistance has become an important problem in terms of broiler farming (6, 9). Although *S.* Infantis, like *S.* Enteritidis, has attracted attention because it is among the serotypes most frequently isolated from poultry farms in many countries in recent years, it is noteworthy that there are few studies in the literature that determine their ability to form biofilms in different environmental conditions (10, 16).

Salmonella strains we used in the research were previously known to be MDR according to EUCAST criteria. Considering that the strains were isolated from poultry litter; the leading risk is that chicken eggs infected with MDR-characterized, biofilm-forming *Salmonella* may enter the food chain and become an important cause of foodborne infections in humans (3).

Salmonella infections are known to spread rapidly. Cross contamination has a great impact on this. In addition, their ability to form biofilms affects their resilience as well as their survival (3). Shen *et al.* (2023) found that 52.78% of *Salmonella* isolates could create biofilms, with the majority being weak biofilm-producing isolates (17). In another study, it was evaluated that 75.86% of all *Salmonella* serovars showed moderate biofilm formation and 24.14% showed strong biofilm formation. Researchers reported that *S.* Enteritidis is the strongest biofilm producer compared to other serotypes (7). Cufaoglu *et al.* (2021) showed that *S.* Infantis strains originating from poultry meat and slaughterhouses are capable of producing strong biofilms (18). Pate *et al.* (2019), reported that 88.5% of the *S.* Infantis isolates were multidrug resistant from broiler flocks and the biofilm-forming capacity of the isolates was generally weak (6). In parallel with the literature, it was determined that the strains used in this study formed biofilms at different degrees (weak, medium, strong).

The aim and approach in finding effective solutions to *Salmonella* infections, which pose an important clinical and industrial problem, is to determine the main factors that are effective during biofilm production. Studies show that biofilm formation plays an important role in the pathogenicity and spread of *Salmonella* (4, 17). There are differences in the amount and ability to form biofilms among *Salmonella* serovars, and these features vary depending on various environmental and genetic factors (19). Considering the optimum living temperatures (37°C), pH (average 7.0) and salt concentration (0%) of bacteria in the *Salmonella* genus, it shows that as *Salmonella* strains go beyond standard living

conditions, they increase their tendency to form biofilms in order to stay infective.

There are different findings in the literature regarding the ideal incubation temperature for biofilm formation. Various studies have reported that low incubation temperature may trigger the biofilm formation capacity of *Salmonella* serovars (11, 20). Another research found that at 5˚C, 44% of the isolates formed weak biofilms and 56% did not create biofilms (21). An additional study reported that *S*. Infantis produced weak biofilm at 4˚C (16). Furthermore, several studies have demonstrated the influence of incubation temperatures of 20˚C, 22˚C, and 37˚C on the biofilm forming ability of *Salmonella* serovars. In a study, the optimum biofilm formation temperature of *S*. Infantis was determined as 20˚C, duration as 72 hours, pH as 6.6 and salt concentration as 0.5% (16). In another study, the strongest biofilm forming capacities of the isolates were detected after three days at 22˚C. Researchers found that at 22˚C, 98% of the isolates (serovars Enteridis, Infantis, Kentucky, and Tel aviv) produced strong biofilms while 2% (serovars Enteritidis) produced weak biofilms (21). Similar to previous studies, it was determined that 72 hours of incubation at 20˚C could be used for strong biofilm production times of all strains.

In a study investigating the biofilm formation potential of *S*. Enteritidis serovar, it was reported that there was a significant difference between 24 and 48 hours of incubation at 22˚C, but there was no significant difference at 30˚C/37˚C and the same incubation periods (11). In another study, determined that the strains formed strong biofilms when the ambient temperature was adjusted to 30˚C and pH 6.0 (12). It was also reported in another study that *S*. Enteritidis and *S*. Infantis serovars could form weak, medium and strong biofilms after a 3-day incubation period at 37˚C (21). In parallel with the studies conducted, it was determined in this study that all strains could be used for strong biofilm production at 37˚C.

It is considered that the difference in data in the literature regarding the biofilm formation potential of *Salmonella* strains may be due to the variability of parameters such as temperature, pH, salt concentration and incubation time, which play a role in the biofilm production of bacteria.

In light of the findings obtained in this study, it has been shown that *S*. Infantis and *S*. Enteritidis serovars are biofilm-producing strains and that their biofilm-forming potential determined at room temperature increases their persistence in poultry habitat. It is clear that the MDR characteristics and biofilm formation abilities identified in *S*. Infantis and *S*. Enteritidis serovars in recent years are parameters that make their eradication difficult. For this reason, investigating the spread and eradication resistance of serovars will be guiding in determining the correct parameters for eradication programs that should be used in industrial, public health and medical fields.

This study did not require ethical approval. The authors declared that there is no conflict of interest.

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