Turkish Journal of Veterinary & Animal Sciences

Volume 49 | Number 2 Article 5

4-21-2025

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YANIK, HAFİZE DİLŞAD; MÜŞTAK, HAMİT KAAN; and SARIÇAM İNCE, SEYYİDE (2025) "Effects of environmental and genetic factors on biofilm formation of various Salmonella serotypes," *Turkish Journal of Veterinary & Animal Sciences*: Vol. 49: No. 2, Article 5. https://doi.org/10.55730/1300-0128.4372
Available at: https://journals.tubitak.gov.tr/veterinary/vol49/iss2/5

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Turkish Journal of Veterinary and Animal Sciences

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Research Article

Turk J Vet Anim Sci
(2025) 49: 70-75
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doi:10.55730/1300-0128.4372

Effects of environmental and genetic factors on biofilm formation of various *Salmonella* serotypes

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Received: 17.04.2024 • **Accepted/Published Online:** 16.02.2025 • **Final Version:** 21.04.2025

Abstract: Biofilm formation by *Salmonella* spp. poses significant challenges across environmental, public health, industrial, and medical sectors, with its capacity to form biofilms influenced by environmental and genetic factors. The present study aimed to identify the biofilm morphotypes of *Salmonella* serotypes (n = 120) and investigate the effects of environmental factors (temperature, pH, and salt concentration) by conventional analysis and genetic factors (*csrB, csrC, fimA,* and *fimI* genes) on biofilm formation by molecular analysis. Five different serotypes of *Salmonella enterica* subsp. *enterica* were used in this study, including serotype Infantis (*S.* Infantis) (n = 100), serotype Enteritidis (*S.* Enteritidis) (n = 5), serotype Hadar (*S.* Hadar) (n = 5), serotype Kentucky (*S.* Kentucky) (n = 5), and serotype Typhimurium (*S.* Typhimurium) (n = 5). Biofilm formation capacities were examined using the microtiter plate method, while the presence of biofilm formation genes was assessed by polymerase chain reaction (PCR) analysis. The analyses indicated that red, dry, and rough (rdar) was the predominant morphotype in *S.* Infantis (96%), *S.* Enteritidis (100%), *S.* Kentucky (100%), and *S.* Typhimurium (100%) serotypes, while brown, dry, and rough (bdar) was found in all *S.* Hadar (100%) isolates. Under suitable conditions, at 20 °C, pH 6.6, and 0.5% salt concentration, the optimal biofilm formation time was 72 h for all serotypes, except for *S.* Typhimurium. No isolates formed biofilms at pH 4.5 or salt concentrations above 0.5%. The biofilm formation genes *csrB, csrC, fimA,* and *fimI,* were found in all isolates (100%). This study revealed the biofilm diversity in *Salmonella* spp. isolates of poultry origin in Türkiye and the effects of environmental factors on biofilm formation. The findings may guide interventions aimed at eliminating *Salmonella* spp. from surfaces by preventing biofilm formation.

Key words: Biofilm, csr operon, environmental factors, genetic factors, poultry, *Salmonella* serotypes

1. Introduction

Salmonella spp. are critical foodborne pathogens that affect humans globally, specifically the second most frequently observed microorganism in foodborne infections. *Salmonella* agents are frequently transmitted through the fecal-oral route into the food chain and contaminates food products [1]. Once a food product is contaminated, *Salmonella* bacteria can retain their viability for extended periods by producing biofilm. This capability enables them to survive on various surfaces and under different conditions of pressure, pH, temperature, etc. [2].

Biofilms are extracellular polymeric matrices synthesized by most bacteria to adhere to external surfaces [3]. Bacteria synthesize biofilm when exposed to challenging environmental factors, such as temperature fluctuations, absence of nutrients, or presence of harmful substances. Biofilm formation protects bacteria from these negative conditions [4]. Biofilm formations are correlated with more than 70% of chronic infections, which increases mortality, morbidity, and treatment costs [5]. Moreover, 50% of nosocomial infections are related to the biofilm formation on medical instruments [6].

Salmonella spp. produce biofilm on both abiotic surfaces (incubators, conveyor belts, steel containers, etc.) and biotic surfaces (fruits, vegetables, eggshells, etc.) [2]. These bacteria have two major components for biofilm formation: cellulose and curli fimbriae. The biofilm structure, which mostly occurs as red, dry, and rough (rdar) morphotype, has been deeply analyzed among the biofilm morphotypes of *Salmonella* agents [3]. This morphotype is responsible for an extracellular matrix that connects bacterial cells on various abiotic and biotic surfaces [7]. Other morphotypes reported for *Salmonella* spp. isolates include brown, dry, and rough (bdar), pink, dry, and rough (pdar), and smooth and white (saw). Biofilm formation starts with the activation of the *csr* and *fim* fimbrial operons, which regulate the biosynthesis of biofilm matrix components [3].

This study aimed to identify the biofilm morphotypes of *Salmonella* serotypes and investigate the effects of environmental and genetic factors on biofilm formation.

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2. Materials and methods

2.1. Bacterial isolates

A total of 120 *Salmonella* spp. isolates were used in this study, consisting of *Salmonella enterica* subsp. *enterica* serotype Infantis *(S.* Infantis) (n = 100), *Salmonella enterica* subsp. *enterica* serotype Enteritidis (*S.* Enteritidis) (n = 5), *Salmonella enterica* subsp. *enterica* serotype Hadar (*S.* Hadar) (n = 5), *Salmonella enterica* subsp. *enterica* serotype Kentucky (*S.* Kentucky) (n = 5), and *Salmonella enterica* subsp. *enterica* serotype Typhimurium (*S.* Typhimurium) (n = 5). These isolates were obtained from various sources, including litter, dust, environmental, rodent traps, feed, and water samples of chicken flocks. They were isolated using the ISO 6579:2002 protocol [8]. Serotyping analysis was conducted using the agglutination test with *Salmonella*-specific antisera (Biorad, France) according to the White-Kauffmann–Le Minor scheme [9]. Additionally, the biofilm producer *S.* Typhimurium ATCC 14028 isolate was used as a positive control in the analyses.

2.2. Detection of biofilm morphotypes

All isolates were inoculated into 5 mL Luria Bertani (LB) broth (Merck, Germany) to obtain 1% dilution. The inoculations were incubated at 37 °C for overnight (at 200 rpm/min). Bacterial cultures were transferred into 5 mL LB broth (without NaCl) (Merck, Germany) before incubation at 37 °C overnight. After incubation, 50 µL of each isolate was transferred onto LB agar (without NaCl) (Merck, Germany) supplemented with 40 µg/mL Congo red (CR) (Sigma Aldrich, USA). CR was preferred as the indicator reagent to visualize the curli fimbriae, as a significant biofilm matrix component [10]. The cultures were incubated at 20 °C for 8 days. This process was repeated twice for each isolate. Biofilm formation was assessed under a stereomicroscope. The isolates were classified into four morphotypes: rdar, bdar, pdar, and saw [3].

2.3. Detection of biofilm capacities

Biofilm capacities of all isolates were detected by the microtiter plate method. For each isolate, the inoculum was adjusted equivalent to McFarland No: 0.5 (1.5×10^8 bacteria/mL) prepared from 1% LB broth dilutions. Approximately 30 µL of each inoculum was placed into a well of the polystyrene microtiter plate consisting of 100 µL LB broth (without NaCl). The microtiter plates were incubated at 20 °C for 24, 48, 72, or 96 h. After incubation, the plates were stained for 15 min with 200 µL of 1% crystal violet (Bacto, Australia), as the indicator reagent for biofilm formation. Approximately 160 µL of 33% glacial acetic acid was used for removing excess stain. Optical density 595 (OD_{595}) was measured using an ELISA reader (BioTek, USA) [11]. Sterile LB broth was used as the negative control sample to define the optical density cut-off (OD_c). In accordance with the Stepanovic method, the spectrophotometric measurements were grouped into different groups: no biofilm formation (non-adherent) for OD ≤ ODc; weak producer (weakly adherent) $OD_c \leq OD \leq 2xOD_c$; intermediate producer (moderately adherent) $2xOD_c \leq OD \leq 4xOD_c$; and strong producer (strongly adherent) OD > 4xODc [12].

2.4. Effects of environmental factors on biofilm formation

The effects of three environmental factors, temperature, pH, and salt concentration, on biofilm formation were investigated. For this purpose, three temperatures (4 °C, 20 °C, and 37 °C), pH values (4.5, 6.6, and 7.3), and salt (NaCl) concentrations (0.5%, 4.5%, and 8%) were adjusted. Different media were prepared and incubated to obtain the required pH values and salt concentrations. Approximately 30 µL of each McFarland No: 0.5 inoculum was placed into a well of the polystyrene microtiter plate containing of 100 µl LB broth (without NaCl). The plates were then incubated to assess the effects of all the investigated parameters [13]. Biofilm capacities were determined as described above.

2.5. Detection of biofilm formation genes

Chromosomal DNA isolation was performed using the Qiagen DNeasy™ DNA extraction kit (Qiagen, USA). DNA samples were used as templates to amplify the *csrB*, *csrC*, *fimA*, and *fimI* genes. Specific primers were designed using BLAST (NCBI) and Primer3 software [14], while polymerase chain reaction (PCR) optimization was performed to determine optimal annealing temperatures (Table). The reactions were adjusted as follows: 1 µL of each 10 µM primer, 2.5 µL 10 × PCR buffer, 3 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP, 0.2 µl of Taq polymerase (2U/µL) (Thermo Scientific, USA), and 2 µL of DNA. Amplifications were conducted at 96 °C for 1 min, 30 cycles of 96 °C for 10 s, 58 °C for 5 s, 72 °C for 4 min, and at 72 °C for 7 min. Positive control (DNA of positive control) and negative control (nuclease-free water) samples were included in all reactions. After agarose gel electrophoresis (1.5%) (Thermo Scientific, USA), the amplicons were examined by a UV transilluminator.

3. Results

As a result of the biofilm morphotype analysis, it was found that 96% of *S.* Infantis isolates exhibited the rdar morphotype, while 4% exhibited the bdar morphotype. All *S.* Enteritidis, *S.* Kentucky, and *S.* Typhimurium isolates (100%) were rdar morphotype, whereas all *S.* Hadar isolates (100%) were bdar morphotype.

The biofilm capacity test revealed that the negative control isolate had an ODc_{595} value of 0.094. During the initial 24 h of incubation, the *S.* Infantis isolates had an OD_{595} value of 0.0567. However, only the positive control isolate (OD_{595} value of 1.2203) and *S.* Typhimurium isolates (mean OD_{595} value of 1.1569) were classified as strong biofilm producers within the first 24 h. Additionally, the *S.* Enteritidis (mean OD_{595} value of 0.0667), *S.* Kentucky (mean OD_{595} value of 0.0587), and *S.* Hadar (mean OD_{595}

Table. Primers used in biofilm gene amplifications.

Gene	Primer sequence (5'-3')	Amplicon size (bp)
csrB	F: CAAGGAAAGGCTTCAGGATG	232
	R: TACTTTTCGTGCTCCCTGCT	
csrC	F: CAAGGAAAGGCTTCAGGATG	217
	R: TACTTTTCGTGCTCCCTGCT	
fimA	F: TGTGCCGTCAGCACTAAATC	181
	R: TGGTGTTATCTGCCTGACCA	
fimI	F: GTCAGCGTTCCATTTTCGTT	217
	R: AAATGGGCGTGTAATGAAGC	

value of 0.0467) isolates were classified as weak producers. Comparing biofilm capacities based on incubation time, the biofilm capacities of the *S.* Typhimurium isolates decreased at 72 h (mean OD_{595} value of 0.4089) whereas the biofilm capacities of the other *Salmonell*a serotypes increased at 72 h (mean OD_{595} values of *S.* Infantis 3.4385, *S.* Enteritidis 3.0667, *S.* Kentucky 3.5087, and *S.* Hadar 3.0467). At 96 h incubation time, biofilm capacities decreased for all serotypes (mean OD_{595} values of *S.* Infantis 2.4225, *S.* Enteritidis 2.0667, *S.* Kentucky 2.5567, and *S.* Hadar 2.0442). Thus, the optimal biofilm formation time was 72 h for all serotypes, except for *S.* Typhimurium (Figure 1).

The optimal temperature for biofilm formation was 20 °C, with only weak biofilm formation at 4 °C and no biofilm formation at 37 °C. The optimal pH for biofilm formation was pH 6.6 for all serotypes, with only weak biofilm formation at pH 7.3 and no biofilm formation at pH 4.4. The optimal salt concentration for biofilm formation was 0.5%, with no biofilm formation at 4.5% or 8% (Figure 2). Finally, all isolates (100%) were found to be positive for the *csrB, csrC, fimA,* and *fimI* genes.

4. Discussion

As predominant foodborne pathogens, *Salmonella* agents are associated with many outbreaks globally. The major source of *Salmonella* transmission to humans is consumption of contaminated poultry products [15]. *S.* Infantis, *S.* Enteritidis, and *S.* Typhimurium are the predominant *Salmonella* serotypes in both humans and broiler chickens [11,16]. Therefore, it is important to identify and characterize biofilm formation in these *Salmonella* serotypes to determine the contribution of biofilm to cross contamination [11].

This study is important in terms of revealing biofilm formation in chicken originated *Salmonella* spp. isolates in Türkiye and the effects of environmental and genetic factors on biofilm formation. The conventional analysis of biofilm formation indicated that rdar was the predominant morphotype in *S.* Infantis (96%), *S.* Enteritidis (100%), *S.* Kentucky (100%), and *S.* Typhimurium (100%) isolates, whereas all *S.* Hadar isolates (100%) were bdar serotype. The fact that all *S.* Infantis isolates exhibited the rdar morphotype, which is known to be more resistant to environmental stress conditions and disinfectants,

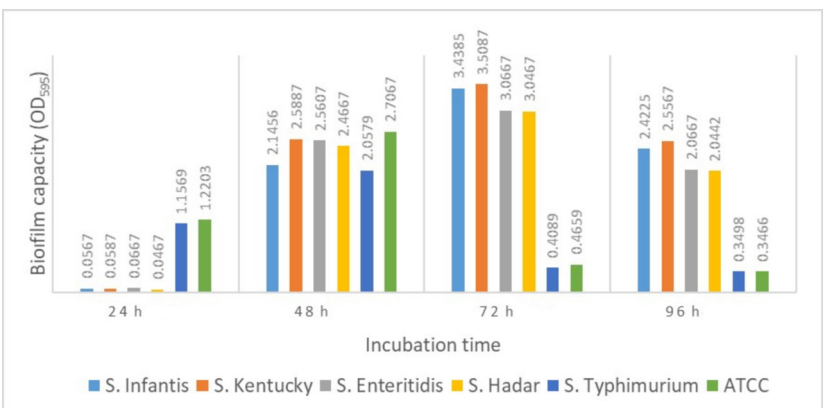

Figure 1. Biofilm capacities of *Salmonella* serotypes based on incubation time.

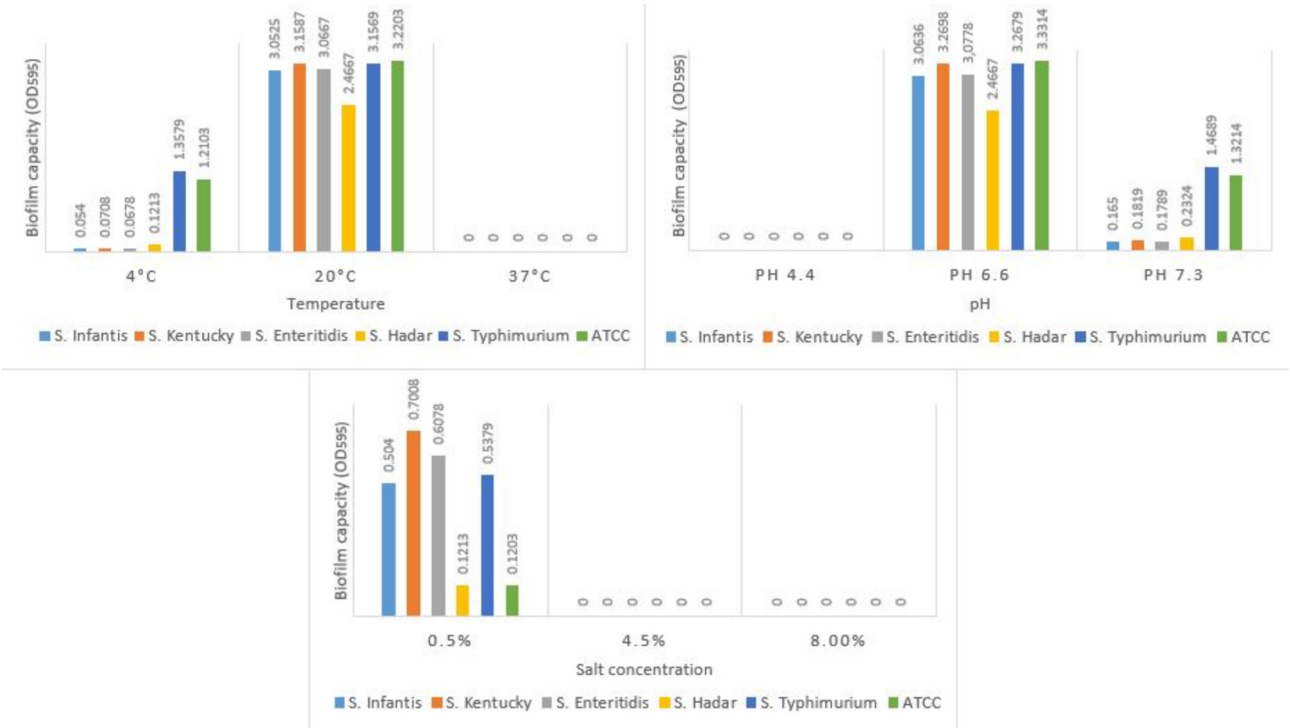

Figure 2. The biofilm capacities of *Salmonella* serotypes based on temperature, pH, and salt concentration.

may explain the high rate of *S.* Infantis isolation from environmental samples in recent years. On the other hand, Olsen et al. [17] reported that all *S.* Typhimurium and *S.* Enteritidis isolates were of the rdar morphotype. In conclusion, our finding of the dominant biofilm morphotype is consistent with previous findings [18,19].

We also investigated how incubation time affected the biofilm capacities of the *Salmonella* isolates. The optimal time for *S.* Typhimurium strains was 24 h, while the optimal time for the other serotypes was 72 h. These findings are similar to those in previous studies reporting that the strongest biofilm capacity of *S.* Typhimurium was at 24 and 72 h whereas that for other *Salmonella* serotypes was only at 72 h [20–22].

Regarding temperature, pH, and salt concentration, the optimum conditions for biofilm formation of the isolates was 20 °C for 72 h, pH 6.6, and 0.5% salt concentration. None of the isolates formed biofilm at 37 °C. These findings are similar to those of previous studies. Lamas et al. [23] reported that *Salmonella* isolates had the highest biofilm formation capacity and rdar morphotype formatting at 20 °C and Lin et al. [24] found that the optimal temperature for *Salmonella* isolates was 20–22 °C, whereas Kumar et al. [25] reported 28–30 °C as the optimal temperature for biofilm formation. Borges et al. [26] reported that no difference on biofilm formations of *S.* Infantis isolates at 3 °C, 12 °C, 28 °C, and 37 °C. On the other hand, Roy et al. [27] reported that higher temperatures from 25 °C to 42 °C caused enhanced biofilm formation of *S.* Kentucky compared to lower temperatures, 4 °C and 10 °C.

The present study revealed that none of the isolates formed biofilm at pH 4.4. Different studies in the literature have reported varying findings regarding the optimal pH for biofilm formation. For instance, Lianou et al. [28] tested the biofilm capacities of 60 *Salmonella enterica* isolates under different environmental conditions and found 53.3% biofilm formation at 25 °C and 58.3% biofilm formation at pH 5.5. These differences can be because we studied different serotypes. Speranza et al. [29] reported strong biofilm capacity at 30 °C and pH 6.0. Giaoruis et al. [30] reported that the optimal pH for biofilm formation was 5.5, while Chavant et al. [31] reported that the optimal pH was 6.5, similar to our finding. Petrin et al. [32] emphasized that most *Salmonella* spp. isolates (including *S.* Typhimurium) were unable to form biofilm at pH 4.5.

In this study, we detected that the optimal salt concentration for biofilm formation was 0.5% for all isolates. None of the isolates formed biofilm at 4.5% or 8% salt concentration. Regarding the effect of salt concentration on biofilm formation in *S.* Enteritidis, our findings were consistent with those of Gu et al. [33]. The authors reported that biofilm formation was significantly inhibited at higher salt concentration than 3%. Contrary to our finding, Petrin et al. [32] reported that *S.* Typhimurium exhibited the strongest biofilm formation at 10% salt concentration.

The last stage of our study examined the presence of *csrB, csrC, fimA, and fimI* genes correlated with biofilm formation in *S*. Infantis, *S*. Typhimurium, *S*. Enteritidis, *S*. Hadar, and *S*. Kentucky isolates. All isolates were positive for all genes (100%). This may be because *csr* and *fim* operons play major roles in biofilm formatting within the *Enterobacteriaceae* family. For example, Altier et al. [34] emphasized the key role of *csr* operon in biofilm formation among strong biofilm producers and nonmutant *S*. Typhimurium isolates. Similarly, Fortune et al. [35] reported that the *csrC* gene regulates the motility-related characteristics of *Salmonella* isolates.

In conclusion, this study indicated that the rdar morphotype was predominant among *S*. Infantis (96%), *S*. Enteritidis (100%), *S*. Kentucky (100%), and *S*. Typhimurium (100%) isolates. However, all *S*. Hadar isolates (100%) exhibited the bdar morphotype. Suitable conditions for biofilm formation were determined to be 20 °C, pH 6.6, and a 0.5% salt concentration for all serotypes. The optimal biofilm formation time was 72 h for all serotypes, except for *S*. Typhimurium. All biofilm formation genes were present in all isolates (100%). The findings of this study may guide interventions aimed at eliminating *Salmonella* spp. from surfaces by preventing biofilm formation.

Acknowledgment

This study was summarized from the master's dissertation of the first author. This study has been supported by Ankara University Scientific Research Projects Coordination Unit under grant number 16L0239014.

Conflict of interest

The authors declare no competing interests.

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