**Original Paper****Biofilm formation and regulating genes of *Salmonella enterica* recovered from chicken and fish samples.**

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**ABSTRACT**

The study sought to evaluate the biofilm formation capacity and biofilm-related gene profiles of *Salmonella enterica* recovered from food of animal origins. A total of 24 *Salmonella enterica* strains were isolated from 215 samples collected from food of animal origins (Raw chicken breast (45 samples), breaded chicken product (45 samples), shrimp (25 samples), Mackerel fish (25 samples), sea bass fillet (25 samples), tilapia fillet (25 samples), and mugil fillet (25 samples)) in Al Qalyubia Governorate, Egypt, during 2020–2022. Using the crystal violet quantitative microtiter plate method and Congo red agar plating, the isolates' ability to form biofilms was evaluated. Polymerase Chain Reaction analysis was used to identify the presence of four biofilm-related genes (*csgA*, *csgD*, *sdiA*, and *sipA*) in strains that were highly capable of forming biofilms. The results revealed that 62.50% (15/24) of the strains exhibited strong biofilm formation, 20.83% (5/24) were moderate biofilm producers, and 16.67 % (4/24) were weak biofilm producers. Over 80% of the *Salmonella enterica* strains that are known to produce strong biofilm were found to be positive for all four of the biofilm-related genes when the strains were analyzed. The current study findings underscored the importance of developing effective preventive and control techniques to address the issues posed by biofilm formation in the food industry.

**1. INTRODUCTION**

Food originating from animals provides substantial sustenance for many people globally; certain negative health effects are associated with their use, such as foodborne pathogens. This places public concerns about the safety of food generated from animals at the top of the list, which is one of the ongoing issues that food producers currently deal with (Edris et al. 2023). *Salmonella enterica*, a harmful bacterium frequently found in food of animal sources, such as chicken, beef, and eggs, is one of the diseases that can be spread by animal food. It causes foodborne infections in people all over the world (Sabeq et al., 2022).

*Salmonella* consists of mainly two species: *Salmonella enterica* (*S. enterica*) and *S. bongori*. Furthermore, molecular characterization and biochemical testing led to the prior classification of *S. enterica* into six subspecies. The main subspecies of *Salmonella enterica* that is under severe threat due to virulent strains that can infect humans and animals with salmonellosis is enterica (Mohammed and Dubie, 2022).

Forming a biofilm is a complex process involving the accumulation of microbial cells and the production of an extracellular polysaccharide matrix (Hajiagha and Kafil, 2023). *Salmonella* biofilm formation is a major concern in food safety and public health. Biofilm formation allows *Salmonella* to persist on food contact surfaces, increasing its resistance to disinfectants and making it more difficult to eliminate (Tee and Abdul-Mutalib, 2023).

Many studies have shown that *Salmonella enterica* strains recovered from food could build biofilms. Biofilm formation by *Salmonella* on foodstuffs has been linked to

food poisoning outbreaks, indicating its significance in foodborne diseases (Baugh et al., 2012). Furthermore, the persistence of *Salmonella* isolates in food and feed manufacturers depends on their biofilm-forming capacities; better biofilm formation correlates with higher persistence (Soni et al., 2013). Additionally, the ability of *Salmonella* to form biofilms along the food chain makes its eradication challenging, highlighting the need to address biofilm formation as a critical factor in food safety (Lamas et al., 2021).

The findings of previous studies emphasize the importance of studying and understanding the biofilm formation of *Salmonella* isolated from food of animal origins, as it directly impacts food safety and the potential for foodborne illness outbreaks. *Salmonella* biofilm formation regulation is a complex process involving numerous genes and pathways. Several studies have identified specific genes that play crucial roles in *Salmonella* biofilm formation (Baugh et al. 2012; Liu et al. 2014; Peng 2016; El Hag et al. 2017).

Understanding the interplay between genes and the impact on *Salmonella*'s behavior is crucial for developing effective strategies to control and mitigate the risks associated with *Salmonella* biofilms. Therefore, the current study aimed at exploring the biofilm formation ability and biofilm-related genes of *Salmonella enterica* isolated from food of animal origins.

**2. MATERIAL AND METHODS****2.1. Study area**

The study was carried out in Al Qalyubia Governorate, Egypt between September 2020 and September 2022 and approved by Research Ethics Committee, Faculty of

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## 2.2. Sample collection and preparation

In Al Qalyubia Governorate of Egypt, 215 animal origin food samples were gathered from markets, supermarkets, and retail and subjected to bacteriological testing to determine whether salmonella was present. Raw chicken breast (45 samples), breaded chicken product (45 samples), shrimp (25 samples), Mackerel fish (25 samples), sea bass fillet (25 samples), tilapia fillet (25 samples), and mugil fillet (25 samples) are among the food of animal origin samples. Within 1h, samples were gathered and brought in sterile plastic bags inside ice box to the laboratory of Food Hygiene and Control, Faculty of Veterinary Medicine, Benha University for bacteriological investigation.

## 2.3. Isolation and Conventional Identification

To isolate and identify salmonella, the standard cultivation procedure suggested by ISO 6579-1: 2017 (ISO, 2017) with some modification was employed. To summarize, 225 milligrams of 10% buffered peptone water were homogenized with a Stomacher 400R (Seward, UK) for two minutes at a weight of ten micrograms per sample. The samples were then incubated overnight at 37 °C in a sterile bag for pre-enrichment. After that, the pre-enrichment was inoculated into 10 mL of Rappaport Vassiliadis (RV) broth (Lab M, UK), and it was left to incubate for 18 to 24 hours at 41°C. Each suspected tube from enrichment broth was streaked onto selective xylose lysine deoxycholate (XLD) agar (BioLife, USA) and incubated at 37 °C for 18 to 24 hours in order to isolate salmonella species. To validate unusual colonies, Bismuth Sulfite Agar (BioLife, USA) was also employed. Using the API 20E® technique (BioMerieux, France), suspected colonies with typical salmonella morphology were biochemically confirmed. salmonella isolates were molecularly confirmed by PCR amplification of a portion of the *invA* gene. The separated bacterial cells were kept in 50% glycerol at –72 °C until they were needed (Central Laboratory, Faculty of Veterinary Medicine, Benha University). Prior to examination, the bacteria were cultivated for 24 hours at 37 °C on Tryptic Soy Agar (TSA) (BioLife, USA). As controls, *Salmonella enterica* ATCC 35664 strains were employed.

## 2.4. Phenotypic assay of biofilm

### 2.4.1. Congo red agar plating

The Congo red agar assay was carried out as described by Cho et al., (2022) with some modifications. Briefly, the bacteria from the overnight broth culture were streaked on Congo Red Agar (CRA) medium using a loopful of inoculum. The CRA medium is made up of tryptic soya agar (TSA) medium plus 3.6% sucrose (Oxoid, UK) and 0.08% Congo red dye (Oxoid, UK), and it is then incubated at 28°C for 48 hours. Colors ranging from brown to black were thought to promote the production of biofilm.

### 2.4.2. Crystal violet (CV) quantitative microtiter plate method

The microtiter plate method was utilized to quantify the production of biofilms by isolating salmonella strains (Hamad et al., 2019). Every isolate of salmonella was cultivated in Brain Heart Infusing (BHI) broth (LabM, UK) for an entire night at 37 °C. Next, 198 µL of BHI was added to 96-well polystyrene microtiter plates that were sterile, and 2 µL of cell suspension was inoculated into each plate. A negative control well holding 200 µL of BHI that has not been infected. After 24 hours at 37 °C, the microtiter plate was incubated. Three gentle washes using 200 µL of phosphate-buffered saline were performed on the wells. The wells had dried up and were inverted. A stain of 0.1% CV (Oxoid, UK) in 125 µL was applied to the biofilm mass. After gently washing the wells three times with 200 µL of distilled water, they were dried upside-down. To solubilize the pigment, the wells were lastly dissolved in 200 µL of 30% acetic acid. At 595 nm, biofilm mass optical density (OD) was measured using a microplate reader (Tecan Sunrise, Jencons, UK). Three standard deviations over the mean OD of the negative control was the definition of the OD cut-off (ODc).  $OD \leq ODc$ , weak biofilm producers ( $ODc < OD \leq 2 \times ODc$ ), moderate biofilm producers ( $2ODc < OD \leq 4 \times ODc$ ), and strong biofilm producers ( $4 \times ODc < OD$ ) were the categories into which all of the isolates were categorized based on their ability to adhere.

## 2.5. Detection of biofilm-regulating genes by PCR (Webber et al., 2019)

To find the biofilm-regulating genes *csgA*, *csgD*, *sdiA*, and *sipA*, PCR analysis was performed on the strong biofilm salmonella producers (n= 15). The QIAamp DNA Mini Kit (Qiagen, Germany) was used to extract the DNA in accordance with the manufacturer's instructions. Table 1 contains a list of all primers and requirements for PCR amplification. To prepare for PCR, a 25 µL reaction mixture was created with 12.5 µL of Emerald Amp GT PCR Master Mix (Takara Bio, Japan) and 1 µL (20 pmol/µL) of each primer (Bio-Basic, Canada), and 6 µL of target DNA. Deionized PCR grade water was used to adjust the remaining volume to reach 25 µL. A T3 Biometra Trio heat cycler (Biometra, Analytik Jena, Germany) was used to carry out the reaction. The following thermal profile was used: The initial denaturation temperature was 94 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min and annealing at 50 °C for 1 minute. (*csgD* gene), 52 °C for 40 sec. (*sdiA* gene), 60 °C for 40 sec. (*sipA* gene) and 57°C for 20 sec. (*csgA* gene); extension temperature at 72 °C for 30 sec (*csgA*, *sdiA* and *sipA* genes), at 72 °C for 45 sec (*csgD* genes); and final extension temperature at 72 °C for 7 min (*csgA*, *sdiA*, and *sipA* gene), and at 72 °C for 10 min (*csgD* genes). The products of PCR (6 µL) were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide, and seen under UV light in a gel documentation system (Alpha Innotech, Kasendorf, Germany).

Table 1. PCR primers and conditions for *Salmonella enterica*-related gene amplification

Target gene	Primer	Sequences (5' to 3')	Amplicon size (bp)	Annealing Temperature	Reference
csgD	F	TTACCGCCTGAGATTATCGT	651	50 °C	(Bhowmick et al., 2011)
	R	ATGTTTAATGAAGTCCATAG			
csgA	F	TCCACAATGGGGCGGCGGCG	350	57 °C	(Webber et al., 2019)
	R	CCTGACGCACCAATTACGCTG			
sipA	F	GGACGCCGCCGGGAAAAAAGTCTC	875	60 °C	(Mezal et al., 2014)
	R	ACACTCCCGTCGCCGCTTCACAA			
sdiA	F	AATATCGCTTCGTACCAC	274	60 °C	(Turki et al., 2014)
	R	GTAGGTAACGAGGAGCAG			

### 2.6. Statistical analysis:

Statistical analysis was performed using STATA version 18.0 (STATA Inc., USA). In order to calculate the biofilm formation outcomes and the gathered data from the various food samples, descriptive statistics like frequency, percentage, and/or proportion were used.

## 3. RESULTS

### 3.1. Isolation and Confirmation of *Salmonella*.

Based on the bacterial morphology, biochemical characteristics, and PCR detection results, 24 suspected salmonella strains were recovered from food samples. The DNA fragment of the *invA* gene, which was utilized to confirm the salmonella genus, was amplified by all strains of salmonella (Figure 1).

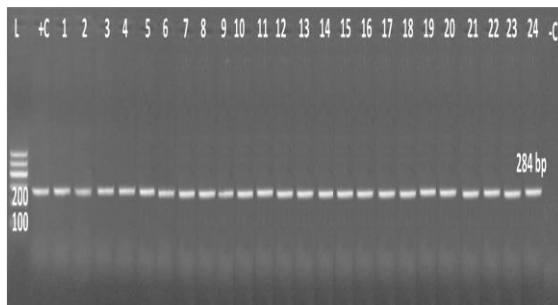


Figure 1: Gel representation of a DNA fragment from the *invA* gene (284 bp) amplified by PCR of representative *Salmonella* strains isolated from food of animal origins. MP-100bp DNA ladder; 1-24: *Salmonella enterica* isolates; 25: negative control *E. coli* ATCC 25922; 26: positive control *Salmonella enterica* ATCC 35664.

### 3.2. Biofilm formation:

The biofilm formation capacities of all the positive *Salmonella enterica* strains were evaluated. Biofilm morphological properties are illustrated in Figure (2). The colonies on Congo Red plates produced curli and cellulose, as seen by their color and appearance. On Congo red agar plates, three distinct colony morphotypes were identified: SAW (Smooth and White), SBAM (Smooth Brown and Mucoïd colony), and RDAR (Red, Dry and Rough). The production of curli and cellulose was indicated by RDAR colonies, the absence of curli and cellulose production was shown by the presence of SAW colonies, and the overproduction of capsular polysaccharide was indicated by SBAM colonies, which showed no cellulose synthesis. According to Figure 2, the morphotypes of RDAR, SAW, and SBAM were prevalent at rates of 54.17%, 20.83%, and 25%, respectively, as confirmed by staining with crystal violet. The findings demonstrated that 24 out of 24 strains, or 100%, had the ability to produce biofilms. As illustrated in Figure 2, of the strains that formed biofilms positively, 62.5% (15/24) were strong producers, 20.8% (5/24) were

moderate producers, and 16.7% (4/24) were poor producers.

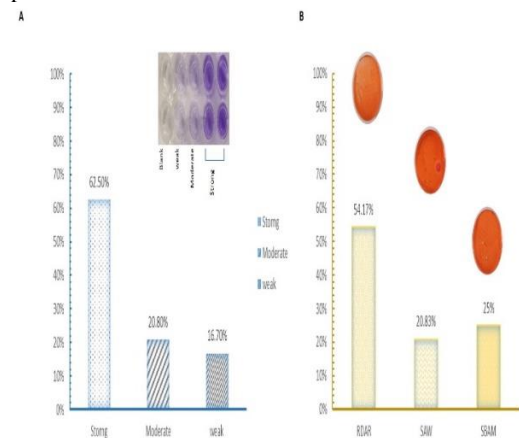


Figure 2: Biofilm formation patterns (A) and morphological characteristics of biofilms (B) of *Salmonella enterica* recovered from food of animal origins.

### 3.3. Biofilm regulates genes.

Among biofilm formation-positive strong producer strains (n=15), the detection rates of the four biofilm-related genes were ranked from high to low as follows: protein (*sipA*), Quorum Sensing (QS) system (*sdiA*), and curli fibers (*csgA*, and *csgD*) (Figure 3).

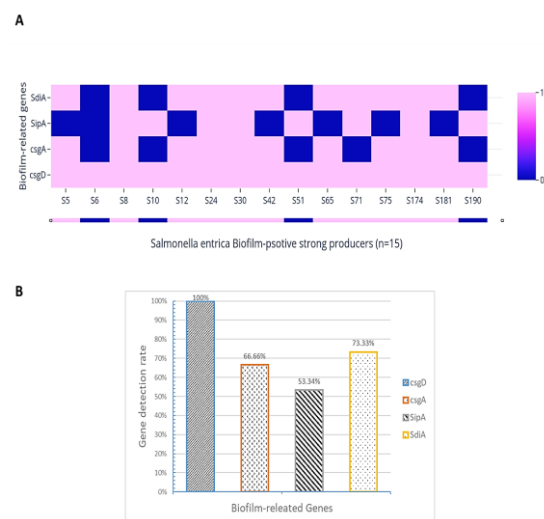


Figure 3: Heatmap of biofilm-related genes (A) (1: for positive; 0: for negative) and gene detection rate (B) of strong biofilm-forming isolates.

Detailed findings are provided, and the PCR results are illustrated in Figures 3 and 4. All strains examined (100%, 15/15) showed detection of all biofilm-related genes, including *csgD*. Of the strains studied, about 66.6% (10/15) tested positive for genes associated with curli fiber (*csgA*). In addition, 73.33% of the *sdiA* gene

was detected (11/15). A protein virulence gene called *SipA* was found at 53.34% (8/15).

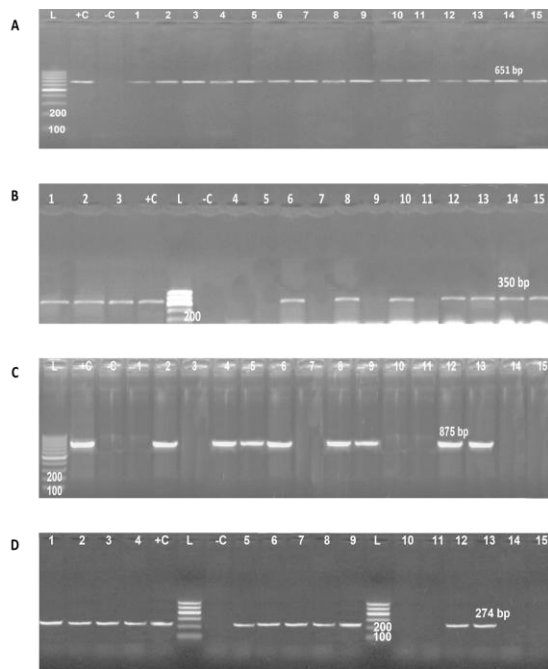


Figure 4: Gel representation of a DNA fragment from (A): *csgD* gene (651 bp); (B): *csgA* gene (350 bp); (C): *sipA* gene (875 bp); (D): *sdiA* gene (274 bp) amplified by PCR; MP-100bp DNA ladder; 1-15: strong biofilm formation isolates; 16: *E. coli* ATCC 25922 (negative control sample); 17: *Salmonella enterica* ATCC 35664 (positive control sample).

#### 4- DISCUSSION

*Salmonella* is a serious problem in foods of animal origin due to its propensity to cause human foodborne diseases. Studies have revealed that *Salmonella* is abundant in animal-derived food products such as meat, eggs, milk, and seafood (Busani et al., 2005; Ejo et al., 2016; Löfström et al., 2004). Because these food products may infect consumers when they eat them raw or undercooked, *Salmonella* poses a health risk to the general public (Chen et al., 2010; Li et al., 2012). Because of its link to antimicrobial resistance, persistence in food animals, and the food chain, *Salmonella* biofilm formation from animal-derived food sources is a serious issue (Koopman et al., 2015). The current investigation demonstrated that *Salmonella* strains have the ability to form biofilms, supporting the conclusions of earlier studies (Nadi et al., 2020; Soni et al., 2013) and posing a risk to food safety. It was established that *Salmonella* might form biofilms (Paytubi et al., 2017).

Genetic determinants are one of the many variables that affect the complex process of *Salmonella* biofilm formation (Chandra et al., 2017; Griewisch et al., 2020). *Salmonella* biofilm development is significantly influenced by the *csgD* gene, which controls the expression of extracellular matrix components associated with biofilms, including cellulose and curli (Ogasawara et al., 2011). According to Liu et al. (2014), this gene is a master transcriptional regulator and a key regulator of *Salmonella* biofilm formation. The results of the current investigation were in line with those of a study on *Salmonella* that forms biofilms and was found in chicken sausages and nuggets. That study's findings strongly imply that cellulose overproduction in *csgD* favorably enhances the development of biofilms (Abdelgwad et al., 2022). Moreover, *csgD* controls a number of genes

essential for the production of biofilms, such as those involved in cellulose manufacturing and cell surface structures (Azam and Khan, 2022).

Additionally, *csgD* controls biofilm formation in agar plates and liquid culture (Grantcharova et al., 2010). Previous research indicates that *Salmonella* biofilm formation is enhanced under low-nutrient circumstances, whereas *csgD* expression is boosted during nutrient deprivation (Paytubi et al., 2017). One of the three main genes implicated in *Salmonella typhimurium* ability to build biofilms, the *csgA* gene is a crucial factor in determining virulence and drug resistance (Baugh et al., 2012). The results of El Hag et al. (2017) and Lozano-Villegas et al. (2023) corroborated our study and demonstrated the importance of the *csgA* gene for public health because of its link to elevated drug resistance in *Salmonella* biofilms.

A multifunctional protein called *Salmonella* invasion protein A (*sipA*) promotes actin polymerization, facilitates bacterial entry into host cells, and modifies actin dynamics—all of which are essential for salmonella biofilm formation (Lilic et al., 2006; Srikanth et al., 2010). The significance of *sipA* in salmonella pathogenicity has been highlighted by the demonstration that it remains active after entry and collaborates with other effectors to control phagosome maturation and intracellular replication (Brawn et al., 2007). The results of Higashide et al., (2002); Jennings et al., (2012); and McGhie et al., (2001) who documented the role of *sipA* in the formation of *Salmonella* biofilm or cell aggregates corroborated the current findings.

The *sdiA* gene is essential for *Salmonella* pathogenicity and biofilm development. When *Salmonella* passes through a turtle's digestive system, the N-acyl-homoserine lactone (AHL) receptor *sdiA* becomes active (Smith et al., 2008). The results of this investigation support the hypothesis that the *sdiA* gene is among the key genes regulating *Salmonella enterica* biofilm formation (Askoura et al., 2021; Michael et al., 2001; Thabit et al., 2022). Because *sdiA* mutants were unable to cling to abiotic surfaces and did not create robust biofilms akin to those formed by wild-type salmonella, the significance of *sdiA* in *Salmonella* adhesion and biofilm development has been highlighted (Elfaky et al., 2022). *Salmonella* gained the gene by the lateral transfer of a pseudomonad homologue to an early ancestor, emphasizing its evolutionary relevance (Nesse et al., 2011).

#### 5. CONCLUSIONS

In conclusion, the biofilm development of *salmonella* isolated from animal foods is an important area of research with direct consequences for food safety and public health. Understanding the biofilm-forming capabilities of *Salmonella* strains from various sources, their persistence, and the obstacles associated with biofilm removal is critical for creating effective prevention and control techniques for foodborne illness. These disparities in biofilm formation ability could be attributable to various variables, including the existence of biofilm formation-related genes, including *csgA*, *csgD*, *SdiA*, and *SipA*. These genes operate as master regulators of biofilm formation in *Salmonella enterica*, triggering matrix component synthesis and enhancing quorum sensing to build biofilm that adheres to surfaces.

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## Data Availability

Supplementary information is available from the relevant author upon justifiable request.

## Competing Interests

The authors claim to have no conflicting of interest.

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