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GENETIC FACTORS RELATED TO THE REGULATION OF BIOFILM FORMATION IN *Salmonella enteritidis* AND *Salmonella typhimurium* IN INDUSTRIAL POULTRY FARMS

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ABSTRACT: The purpose of the study was to examine the genetic mechanisms and regulation of biofilm formation regulation in *Salmonella enteritidis* and *Salmonella typhimurium* isolated at industrial poultry farms. The methodology included the collection of 340 samples from industrial poultry production facilities in Kazakhstan, Latvia, and Turkey between 2022 and 2025. The isolated strains were serotyped, genomic deoxyribonucleic acid was extracted, and the presence of the *csgD*, *bcsA*, *adrA*, *rpoS*, and *fimA* genes was detected using polymerase chain reaction (PCR). The level of gene expression was determined using quantitative polymerase chain reaction, and the phenotypic ability to form biofilms was evaluated by crystal violet staining. The results showed the isolation of 238 *Salmonella* strains, including 124 *S. enteritidis* and 114 *S. typhimurium*. The highest contamination was recorded in slaughter lines, accounting for 43.3% of the total positive samples. The analysis showed varying *Salmonella* serotype prevalence across countries, with *S. enteritidis* dominant in Kazakhstan (45.9%), *S. typhimurium* in Latvia (64.7%), and a balanced distribution in Turkey. The analysis revealed a high prevalence of biofilm formation genes, particularly *fimA* (94.1%), while *rpoS* ranged from 66.7% to 85.5%. According to quantitative polymerase chain reaction data, the expression of *csgD* (p=0.003), *bcsA* (p=0.005), and *adrA* (p=0.007) was significantly higher in *S. typhimurium*, indicating its greater potential for biofilm formation. The phenotypic assessment confirmed this: 65% of *S. typhimurium* were strong or very strong biofilm producers, compared with 45% in *S. enteritidis* (OD570: 1.42±0.15 and 0.97±0.12, respectively). The results can be practically applied in the development of sanitary control programmes, including the implementation of protocols for periodic assessment of biofilm-forming strains using qPCR screening. Based on the expression profile of key genes, criteria can be developed for selecting effective disinfectants (e.g., peroxide compounds or quaternary ammonium compounds) and for creating regulations targeting critical areas, such as poultry slaughterhouses and evisceration equipment.

Keywords: Phenotypic variability, Poultry farming, Regulatory mechanisms, Serotypes, Stress response.

INTRODUCTION

The development of poultry farming, as one of the most rapidly expanding branches of the agro-industrial complex, is accompanied by increased biological and sanitary risks associated with microbial contamination of products (Abreu et al., 2023; Sychov et al., 2024). *Salmonella* bacteria, which are highly adaptable and capable of long-term persistence in the environment, pose a particular threat to food chain safety (Turmagambetova et al., 2017; Mussayeva et al., 2023). For example, Ehuwa et al. (2021) emphasize that *Salmonella* remains a persistent public health concern due to its ability to survive under diverse environmental conditions and contaminate a wide range of foods. Similarly, Mkangara (2023) highlights the role of biofilm formation in poultry-processing plants, showing that biofilms protect *Salmonella* from sanitation and antimicrobial measures, thereby facilitating its persistence and transmission through the food chain. Biofilm formation is therefore a crucial factor in the survival of these microorganisms in the production lines of poultry processing plants, providing them with resistance to sanitation, antimicrobials, and physical influences. Biofilm formation is therefore a crucial factor in the survival of these microorganisms in poultry production lines. as it provides resistance to sanitation, antimicrobials, and physical influences (Berezin et al., 2008; Obe et al., 2021). Among the various serotypes, *Salmonella enteritidis* and *Salmonella typhimurium*, which are widespread in industrial poultry farming and possess a pronounced pathogenic potential for humans, are considered particularly dangerous (Demyanyuk et al., 2023; Shaji et al., 2023).

An important feature of the poultry processing plants environment is the constant presence of stress factors, including temperature fluctuations, the use of disinfectants, and the mechanical stress, as noted by Ncho et al. (2024). These conditions contribute to the activation of bacterial stress responses, which in turn can enhance biofilm formation. Mendybayeva et al. (2023) demonstrated in an experimental model that exposure to hypo- and hyperosmotic conditions increases the expression of regulatory genes, including *rpoS* and *csgD*, which are associated with the synthesis of matrix components and greater resistance of *Salmonella* to environmental influences at various stages of the technological and

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industrial process. However, their study did not address differences in gene expression between serotypes, nor did it include an extended molecular genetic analysis to identify specific pathways regulating the stress response.

The formation of a mature biofilm largely depends on the ability of bacteria to produce cellulose and protein components of the matrix (Coutinho et al., 2016; Aipova et al., 2020). It has been established that the *csgD*-dependent regulatory system is the key controller of this process; however, data on the serotype-specifics of mechanisms of its activation under industrial conditions remain limited. Dančová et al. (2024) reviewed the role of the *csgD* gene in the regulation of amyloid fibre production in *Salmonella enterica*, demonstrating its importance for the initial stages of biofilm formation through quantitative PCR and evaluation of colony morphology on Congo Red agar. However, their study did not include a comparative analysis of *csgD* expression levels among different serotypes, leaving open the question of serotype-specific regulatory mechanisms.

A serious challenge in poultry processing is the high variability in the biofilm forming ability of different *Salmonella* strains, which complicates risk forecasting (Pyatkovskyy, 2023; Adamchuk and Voinalovych, 2024). Arkali and Çetinkaya (2020) demonstrated substantial differences in the intensity of biofilm formation among isolates from various industrial facilities (equipment surfaces and cooling baths) using the crystal violet staining method. However, their study did not include parallel molecular genetic analyses (such as the expression of *csgD*, *adrA*, and *bcsA* genes), which could have identified the molecular determinants underlying the observed phenotypic variability.

Intracellular signal transmission systems, particularly, the cyclic di-GMP system, are fundamental to the regulation of biofilm formation. It is well established that an increase in the level of this second messenger stimulates the synthesis of exopolysaccharides and promotes biofilm development. İnce and Akan (2023) investigated the effect of mutations in cyclic di-GMP synthase genes on biofilm production in laboratory *Salmonella* strains, revealing a substantial decrease in biofilm activity. However, such studies have rarely been conducted on isolates obtained directly from industrial poultry facilities.

The regulation of the stress response mediated by the sigma factor *rpoS* is also recognised as a critical element for bacterial survival under harsh conditions (Bouillet et al., 2024; Abutalip et al., 2025). Increased *rpoS* expression enhances resistance to disinfectants and promotes biofilm formation. Rychshanova et al. (2021) demonstrated the major role of this gene under oxidative stress; however,their study was limited to model systems and did not include industrial isolates, which restricts the practical applicability of their findings. An important factor in the initial stages of bacterial attachment to surfaces is the activity of fimbrial structures (Montayev et al., 2023; Ospanov et al., 2024). The *fimA* gene encodes the main subunit protein of type-1 fimbriae, which mediate primary cell adhesion. Liu et al. (2022) showed that type-1 fimbriae gene regulate cell adhesion and amino acid excretion, providing insights into biofilm-based fermentation in *E. coli*. Zhanabayeva et al. (2021) reported the high conservation and prevalence of the *fimA* gene among various *Salmonella* serotypes; however, their study did not examine gene expression under conditions simulating poultry processing environments.

Recent studies, such as Ban-Cucerzan et al. (2025), have also highlighted the role of environmental conditions in modulating bacterial biofilm properties. Commonly, high levels of organic residues and the presence of microdefects on equipment surfaces create favourable conditions for the establishment and development of biofilm communities. Chen et al. (2021) demonstrated that contamination of slaughter lines is directly correlates with the intensity of *Salmonella* biofilm formation; however, their study did not examine the relationship between these observations and molecular and genetic characteristics of the strains.

Insufficient knowledge of the regional specificity of *Salmonella* serotypes in relation to their biofilm-forming activity remains a significant problem. Given the differences in technological processes and sanitary standards across countries, these aspects are particularly important. Sharma et al. (2022) reported that the geographical origin of isolates influences their resistance levels and biofilm-forming ability; however, their conclusions were based primarily on epidemiological data without a detailed molecular interpretation of the underlying mechanisms.

Thus, the studies reviewed above highlight the importance of a comprehensive analysis of the genetic regulators of biofilm formation in *Salmonella enteritidis* and *Salmonella typhimurium* serotypes circulating in industrial poultry farms. The study aimed to identify the molecular mechanisms regulating biofilm formation in *Salmonella enteritidis* and *Salmonella typhimurium* isolated from industrial poultry facilities, thereby determining the characteristics of their biofilm activity and resistance of environmental stresses. The objectives of the study included assessing the prevalence of genes responsible for biofilm formation, analysing their expression levels in different serotypes, and phenotypically evaluating the ability to form biofilms under conditions that simulate poultry production processing environments.

MATERIALS AND METHODS

Study design and sampling

The study was conducted between 2022 and 2025 at the Kazakh National Research Agrarian University in cooperation with the industrial poultry farms of Kazakhstan, Latvia, and Turkey. Typical poultry farms with both cage and outdoor systems were selected as sites, differing in production scale (ranging from small to large farms) and biosafety level (from basic to advanced, according to the internal protocols of the enterprises). Sampling was carried out

purposefully, taking into account the technological role of each facility and the potential risk of contamination. The sample included production sites where broiler and laying hens were raised; facilities with incomplete sanitary documentation were excluded. Samples were collected from poultry houses, incubators, and slaughtering lines, including equipment surfaces, watering systems, litter transportation belts, and interior walls. The flushing method with sterile swabs (Copan Diagnostics Inc., USA) placed in a transport medium (Amis Inc., USA) was used, followed by delivery to the laboratory in refrigerated containers at +4 °C within no more than 24 hours. All sampling was conducted in compliance with the sanitary regulations in force in each country to minimize cross-contamination and ensure the representativeness of the results.

Isolation and identification of *Salmonella* strains

Primary isolation of *Salmonella* spp. was performed on Buffered Peptone Water and Rappaport-Vassiliadis Soy Peptone Broth enrichment media (Oxoid Ltd., UK), followed by planting on selective media (XLD Agar and CHROMagar *Salmonella*, France). The isolates were serotyped using a set of agglutination sera (Denka Seiken, Japan) according to Kaufman-White scheme. All isolated strains of *Salmonella enteritidis* and *Salmonella typhimurium* were stored in a strain collection on Tryptic Soy Broth supplemented with glycerin at -80°C.

Genomic DNA Extraction and PCR Analysis

Genomic DNA was extracted from isolates using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, USA) to analyse genetic factors regulating biofilm formation. DNA concentration and purity were determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and further verified by electrophoresis in 1% agarose gel stained with SYBR Safe dye (Thermo Fisher Scientific, USA). The *csgD*, *bcsA*, *adrA*, *rpoS*, and *fimA* genes responsible for cellulose synthesis, curl formation, and stress response regulation were detected using PCR on a T100 Thermal cycler (Bio-Rad, USA). Specific oligonucleotide primers were designed based on NCBI data and synthesised by Integrated DNA Technologies (USA). Amplification conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 56 - 60°C for 30 seconds (depending on the target gene) and elongation at 72°C for 1 minute, with the final extension at 72°C for 7 minutes. Amplification specificity was confirmed by electrophoresis in a 1.5% agarose gel.

Quantitative Real-Time PCR

Expression level of biofilm-associated genes were analyzed by quantitative real-time PCR (qPCR) using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) with SYBR Green Master Mix (Thermo Fisher Scientific, USA). The 16S rRNA gene was used as the internal control for normalization, and relative expression levels were calculated using the ΔΔCt method.

Phenotypic Assessment of Biofilm Formation

The biofilm-forming ability of isolates was phenotypically assessed using crystal violet staining on 96-well polystyrene plates (Nunc, Denmark). Each sample was incubated for 48 hours at 28°C in Luria-Bertani broth (Oxoid Ltd., UK) supplemented with 0.2% glucose to stimulate biofilm development. After incubation, biofilms were fixed with methanol, stained with 0.1% crystal violet solution, and solubilized with 95% ethanol. Optical density was measured at 570 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, USA).

Statistical analysis

Statistical data processing was performed in GraphPad Prism 9 software, USA (GraphPad Software, 2020). The Shapiro-Wilk criterion was used to verify the normality of the data distribution. Intergroup differences were assessed using the Student's t-test to measure normally of the distributed data and the Mann-Whitney test was performed to detect abnormal samples. The level of statistical significance was considered as $p<0.05$.

RESULTS

As a result of the study, 238 strains of *Salmonella* bacteria were isolated in industrial poultry farms in Kazakhstan, Latvia, and Turkey, of which 124 belonged to the *Salmonella enteritidis* serotype, and 114 to *Salmonella typhimurium* (Table 1). The highest number of isolates was recorded in Kazakhstan (98 positive samples), with *S. enteritidis* being the dominant serotype. In Latvia, 76 isolates were obtained, with a predominance of *S. typhimurium*, particularly on slaughter lines. In Turkey, 64 isolates were identified, with an approximately equal distribution between the two serotypes. Among the sampling sites, slaughter lines showed the highest level of contamination (43.3% of the total number of positive samples), while incubators had the lowest occurrence of *Salmonella* spp. (18.9%). These findings highlight the importance of comprehensive monitoring at all stages of poultry production.

Table 1 - Distribution of isolated strains of *Salmonella* spp. by country and technological facilities

Country	Object	Number of samples	*S. enteritidis*	*S. typhimurium*	Total positives
Kazakhstan	Poultry house	60	22	14	36
Kazakhstan	Incubator	40	9	7	16
Kazakhstan	Slaughter line	40	20	26	46
Latvia	Poultry house	50	10	15	25
Latvia	Incubator	30	5	7	12
Latvia	Slaughter line	40	7	32	39
Turkey	Poultry house	50	14	10	24
Turkey	Incubator	30	8	6	14
Turkey	Slaughter line	40	9	17	26
Total	-	340	104	134	238

*Source: compiled by the authors.

The analysis of the obtained data demonstrated clear differences in the prevalence of *Salmonella enteritidis* and *Salmonella typhimurium* serotypes across the countries and technological facilities of poultry enterprises studied. In Kazakhstan, the dominant serotype was *S. enteritidis* (45.9% of all isolates), which corresponds to previously identified trends in the spread of this pathogen in regions with large industrial poultry farms. In Latvia, by contrast, *S. typhimurium* was detected more frequently (64.7%), particularly on slaughter lines, suggesting its possible dissemination through equipment and transport belts. In Turkey, the distribution of serotypes was relatively balanced, which may reflect similar transmission mechanisms or characteristics of local poultry farming practices.

The distribution of *Salmonella* spp. among different sampling sites also revealed substantial differences. The highest number of positive samples was recorded on slaughter lines (43.3% of the total number of isolates), likely due to the high degree of equipment contamination, challenges in disinfection, and cross-contamination of birds during processing. The finding is particularly supported by the high proportion of *S. typhimurium* in Latvia (82.1% of slaughter samples), consistent with the hypothesis that this serotype can persist and spread within the meat processing facilities. In contrast, incubators showed the lowest level of contamination (18.9%), which may be attributed to controlled conditions, regular sanitation, and the absence of faecal pollution.

Additional analysis of geographical differences demonstrated that the higher detectability of *S. enteritidis* in Kazakhstan may be attributed to its historically high prevalence among laying hens and the limited effectiveness of existing biosafety protocols. In contrast, in Latvia, the more frequent detection of *S. typhimurium* on slaughter lines supports the hypothesis of stable reservoirs in meat processing plants, highlighting the need for more rigorous disinfection measures. In Turkey, the approximately equal ratio of serotypes may suggest combined sources of infection, including feed, bedding, and equipment.

Statistical analysis confirmed significant differences in serotype prevalence between countries ($p < 0.05$), particularly in the “slaughter lines” and “poultry houses” groups. The prevalence of *S. enteritidis* in Kazakhstan was statistically higher ($p = 0.03$) compared to other countries, while S. typhimurium dominated in Latvia ($p = 0.02$). In Turkey, no statistically significant differences were observed between serotypes ($p = 0.07$), which may indicate a high level of circulation of both pathogens within the country’s poultry systems.

These results emphasize the need for enhanced monitoring of *Salmonella* spp. in slaughterhouses and processing facilities, as these represent critical points in the pathogen transmission chain. The observed differences between countries may reflect the influence of local factors, such as poultry management systems, disinfection practices, and food safety controls. A molecular study of *S. enteritidis* and *S. typhimurium* isolates further revealed a high prevalence of key genes associated with biofilm formation (Table 2). The analysis focused on five target genes—*csgD, bcsA, adrA, rpoS, and fimA*—each of which plays an essential role in synthesizing structural biofilm components, regulating stress responses, and enhancing bacterial resistance to environmental factors.

Table 2 - Percentage of positive isolates for detection of *csgD*, *bcsA*, *adrA*, *rpoS*, and *fimA* genes (%)

Country	Keep condition	*csgD*	*bcsA*	*adrA*	*rpoS*	*fimA*
Kazakhstan	Cage	91.2%	88.2%	85.3%	80.9%	94.1%
Kazakhstan	Outdoor	86.3%	82.4%	78.4%	74.5%	92.2%
Latvia	Cage	85.7%	80%	77.1%	72.9%	93.3%
Latvia	Outdoor	83.3%	77.8%	70.4%	66.7%	88.9%
Turkey	Cage	92.1%	90.8%	88.2%	85.5%	95%
Turkey	Outdoor	90%	87.5%	85%	81.3%	93.8%
Average	-	88.7%	85.3%	81.5%	77.3%	94.1%

*Source: compiled by the authors.

Analysis of the prevalence of key genes regulating biofilm formation in isolates of *Salmonella enteritidis* and *Salmonella typhimurium* revealed clear patterns reflecting the complexity and multi-layered genetic control of this process. All five examined genes showed a high detection rate, which confirms the wide representation of biofilm formation mechanisms in *Salmonella* spp. populations on poultry farms.

The *fimA* gene was identified as the most consistently detected in almost all isolates. This gene encodes a subunit of type 1monomeric fimbriae, which mediates the initial stage of bacterial cell adhesion to abiotic surfaces. The high prevalence of *fimA* highlights the fundamental role of fimbriae in colonising the environment and triggering biofilm formation. Type 1 fimbriae are involved not only in primary attachment but also in subsequent cell aggregation, contributing to the development of dense multilayered structures.

The *csgD* gene showed a slightly lower, though still high detection rate. As a central regulator of biofilm formation, *csgD* controls the synthesis of amyloid fibrils and initiates cellulose production. Its expression product activates a cascade of signalling pathways, including two-component regulatory systems, enabling the bacteria to transition to a biofilm state. The high prevalence of this gene confirms its pivotal role in switching *Salmonella* spp. between planktonic and the attached lifestyle, which is critical for their long-term persistence under the harsh conditions of poultry farms.

The *bcsA* and *adrA* genes, which control the synthesis of cellulose – the main component of the extracellular biofilm matrix, showed high but somewhat variable prevalence. *bcsA* encodes cellulose synthase, which is directly responsible for the polymerisation of β-1,4-glucans, while *adrA* regulates the activity of the Bcs complex by producing cGMP, thereby enhancing cellulose synthesis in response to external signals. The high detection rate of these genes confirms the active involvement of isolates in forming stable three-dimensional biofilm structures with a dense matrix capable of protecting cells from antiseptics and antibiotics.

The *rpoS* gene, which encodes the sigma factor of the general stress response, was characterised by the greatest variability. Its lower detection rate may reflect the dynamic regulation of this element depending on environmental conditions. *rpoS* activates the expression of a wide range of stress-associated genes, including those involved in biofilm formation, and regulates the transition to the stationary growth phase. The observed variability in this gene suggests that not all isolates possess the same capacity to activate universal defence mechanisms under stress, which directly influences their survival in harsh production environments.

Collectively, the high prevalence of the *fimA*, *csgD*, *bcsA*, and *adrA* genes demonstrates the strong genetic potential of most isolates for biofilm formation. The variability in *rpoS* highlights differences in stress tolerance among strains. These findings confirm the complex nature of biofilm formation in *Salmonella* spp., which involves both structural and regulatory components. The presence of such intricate genetic systems indicates that biofilms are essential elements for *Salmonella* in poultry production, protecting bacteria from external factors and increasing the risk of product contamination. Quantitative PCR analysis was used to determine the relative expression levels of the *csgD*, *bcsA*, *adrA*, *rpoS*, and *fimA* genes in isolated strains of *Salmonella enteritidis* and *Salmonella typhimurium*. All genes showed differential activity between the two serotypes, reflecting the serotype-specific of biofilm formation at the transcriptional level (Table 3). The average expression level of the key regulatory gene *csgD* was higher in *S. typhimurium*, which indicates a more active activation of the signalling pathways of biofilm formation in this serotype. The *bcsA* and *adrA* genes, which control cellulose synthesis, also showed higher transcriptional activity in *S. typhimurium*, which confirms its potentially greater ability to form a dense biofilm matrix.

Table 3 - The average relative level of gene expression (ΔΔCt) in *Salmonella enteritidis* and *Salmonella typhimurium*

Gene	*S. enteritidis*	*S. typhimurium*	p-value
csgD	1.85±0.14[b]	3.12±0.18[a]	0.003
bcsA	1.62±0.1[b]	2.75±0.15[a]	0.005
adrA	1.51±0.09[b]	2.48±0.13[a]	0.007
rpoS	1.33±0.12[b]	1.89±0.11[a]	0.021
fimA	2.05±0.17	2.22±0.19	0.157

Note: Values with different letters (a, b) within the same row indicate statistically significant differences ($p<0.05$). The highest value in each row is marked with "a". Source: compiled by the authors.

Gene expression analysis of *csgD*, *bcsA*, *adrA*, *rpoS*, and *fimA* showed clear differences between the serotypes of *Salmonella enteritidis* and *Salmonella typhimurium*, presenting the features of the molecular regulation of biofilm formation in each of them. The most pronounced differences were recorded in the *csgD* gene, which acts as a central transcriptional regulator of biofilm formation. Substantially higher *csgD* expression in *S. typhimurium* indicates increased activation of signalling pathways that trigger extracellular matrix synthesis and switch cells to a biofilm lifestyle. This regulator controls the downstream expression of a number of structural genes and thereby determines the ability of the bacterial population for long-term attachment and survival in the harsh environment.

The *bcsA* and *adrA* genes responsible for the synthesis of cellulose - one of the main components of the extracellular matrix - demonstrated similar trends. Substantially higher transcriptional activity of *bcsA* and *adrA* in *S. typhimurium* proves its genetic predisposition to the synthesis of dense and stable biofilms with a developed structural matrix. The high level of *bcsA* expression indicates the active work of the cellulose synthase complex, capable of producing a substantial amount of β-1, 4-glucans, which enhances the mechanical strength of the biofilm. Therewith, *adrA* activates cGMP synthesis and enhances cellulose production, forming a closed regulatory loop that enhances the biofilm properties of the population. The expression of the *rpoS* gene, responsible for activating stress responses and the transition of cells to the stationary growth phase, was also higher in *S. typhimurium*. This result indicates a greater ability of this serotype to activate universal protective mechanisms in response to adverse environmental factors. Increased *rpoS* transcription correlates with potentially higher *S. typhimurium* resistance to environmental conditions, including exposure to disinfectants and other stressors during production. The analysis of *fimA* expression was considered separately. Although the gene is traditionally regarded as the most important factor of initial adhesion, its expression levels were nearly identical in both serotypes. This finding highlights the universality of type 1 fimbriae during the early stages of attachment and their relatively conserved regulation, independent of serotype.

Overall, the results demonstrate that *S. typhimurium* exhibits a higher level of transcriptional activation of the main biofilm formation genes, providing an advantage in the formation of denser, more stable and viable biofilms. This serotype is potentially dangerous in terms of long-term persistence on equipment surfaces and in the environment of poultry farms. *S. enteritidis* showed less pronounced activity in most genes, which may indicate a slightly weaker biofilm potential compared to *S. typhimurium*. Nevertheless, the high activity of *fimA* in both serotypes confirms the presence of a basic level of adhesive properties sufficient to initiate colonisation and subsequent spread.

A phenotypic assessment of biofilm formation by crystal violet staining revealed varying degrees of biofilm production among the isolates of *Salmonella enteritidis* and *Salmonella typhimurium*. The isolates exhibited a wide range of optical density values (OD_{570}), reflecting differences in their biofilm-forming potential (Table 4). Based on these measurements all strains were classified into four groups: weak, moderate, strong, and very strong biofilm-forming agents. The largest proportion of strong and very strong biofilm-forming agents was found among *S. typhimurium*, where the number of isolates with high OD_{570} exceeded 65%. *S. enteritidis* showed more moderate activity, with about 45% of the isolates classified as strong and very strong biofilm producers.

Table 4 - Distribution of isolates by biofilm formation level (optical density OD_{570}, M±m)

Serotype	Average OD_{570} (M±m)	Weak (%)	Moderate (%)	Strong (%)	Very strong (%)
S. enteritidis	$0.97±0.12^{b}$	18%	37%	28%	17%
S. typhimurium	$1.42±0.15^{a}$	8%	27%	35%	30%

*Different superscript letters within the same column indicate statistically significant differences ($P<0.05$). For average OD570 values, $p=0.032$. Source: compiled by the authors.

The phenotypic assessment of the ability of *Salmonella enteritidis* and *Salmonella typhimurium* isolates to form biofilms demonstrated substantial differences in the level of biofilm activity between the serotypes. The classification based on optical density indicators (OD_{570}) revealed a clear trend towards a more pronounced biofilm-forming ability in *S. typhimurium*, consistent with previously obtained molecular results.

The proportion of strong and very strong biofilm-forming agents among *S. typhimurium* reached 65%, while *S. enteritidis* had a rate of only 45%. This difference highlights the higher potential of *S. typhimurium* in the formation of mature and dense biofilms capable of providing long-term bacterial persistence on abiotic surfaces. The average value of OD_{570} in *S. typhimurium* was 1.42±0.15, substantially higher than that of *S. enteritidis* (0.97±0.12), thereby confirming a more active accumulation of biomass and the intensity of matrix formation.

The observed high biofilm activity of *S. typhimurium* is primarily explained by the increased expression of regulatory and structural genes that control extracellular matrix synthesis. This ensures not only the strong fixation of bacterial cells on surfaces but also the formation of a protective barrier that can effectively reduce the penetration of antiseptics and other disinfectants. These features lead to conditions for the survival of *S. typhimurium* even during aggressive sanitary treatment of industrial premises. Therewith, *S. enteritidis* showed a more moderate biofilm-forming activity, which may indicate its slightly lower ability to form long-term fixation and persistence. Nevertheless, even with relatively lower OD_{570} values, about 45% of isolates of this serotype were classified as strong and very strong biofilm-forming agents, confirming the potential danger of this pathogen in poultry processing plants. High biofilm activity of individual *S. strains. enteritidis* strains can be explained by the presence of favourable regulatory mutations in them or by the activation of alternative synthesis pathways of extracellular matrix components.

The results indicate that the phenotypic ability to biofilm is the most important characteristic of the virulence and resistance of *Salmonella* spp. The high density and structural organisation of biofilms provide both physical protection of

cells and contribute to the horizontal transfer of resistance genes, which increases the risk of the formation of multidrug-resistant strains in an industrial environment. Consequently, the presence of a large number of strong biofilm-forming agents among *S. typhimurium* isolates poses a serious threat to the sanitary safety of poultry farms and requires constant monitoring and the development of targeted strategies for the destruction of biofilms in technological cycles.

DISCUSSION

This study established substantial differences in the prevalence of *Salmonella enteritidis* and *Salmonella typhimurium* serotypes in poultry farms in Kazakhstan, Latvia, and Turkey, and to identify critical contamination points at various stages of the production cycle. The findings confirm the importance of comprehensive monitoring of pathogens in the industry, which is consistent with the conclusions of Badie et al. (2021), who emphasized the need to account for regional and technological factors in the spread of *Salmonella* in poultry processing complexes.

The revealed predominance of *S. enteritidis* is particularly notable in Kazakhstan, which reflects the steady consolidation of this serotype in the poultry practice of the region. Similar patterns were observed in a study by Pradhan et al. (2023), which underlined the relationship between the characteristics of poultry keeping and the dominance of *S. enteritidis*. However, in contrast to these data, Chen et al. (2023) reported the predominance of *S. typhimurium* in similar conditions. This discrepancy may be explained by differences in the genetic composition of poultry herds and the biosafety programmes used, which confirms the more objective nature of this study (large number of samples).

High frequency of *S. typhimurium* detection in Latvian slaughterhouses demonstrates the presence of stable contamination reservoirs in processing plants, which underlines the importance of this stage of the production process as a critical control point. The highest number of positive samples on slaughter lines indicates a high probability of accumulation of pathogens on equipment surfaces, transport belts and in hard-to-reach areas where disinfection is difficult. Similar conclusions are presented in the work of Holden et al. (2022), underscoring the importance of equipment in the transmission of pathogens and pointing to the role of micro-lesions of the surface and biofilms in the accumulation of bacteria. Ma et al. (2025), on the contrary, argued the minimal role of slaughter lines in the spread of *Salmonella* spp., which is not confirmed in this study, while the processing stage was uncovered to be the most problematic and required special attention from sanitary control. This highlights the need for enhanced monitoring of equipment conditions and a review of standard sanitation procedures at slaughterhouses to prevent further spread of infection.

The relatively uniform distribution of serotypes in Turkey deserves special attention, which is likely due to the specific features of the industrial environment and the combined sources of infection. This finding may reflect the influence of various factors, including heterogeneous sanitary practices, the use of feed from different origins, and the possible introduction of pathogens from the environment. Such conditions promote the simultaneous circulation of several serotypes, thereby complicating the development of targeted prevention and control measures. Kao et al. (2023) reported similar results, emphasizing the complex role of feed, litter, and equipment in maintaining the circulation of multiple serotypes at the same time. In contrast, a study by Siddique et al. (2021) highlighted the dominance of a single serotype under similar conditions, a finding not supported by the present study. Furthermore, the observed uniformity in serotype distribution may indicate the need for more detailed monitoring programmes that account for the variety of possible contamination sources and their contributions to the epidemiological situation in enterprises.

Analysis of the molecular data revealed a high prevalence of genes responsible for biofilm formation in isolates of both serotypes. Fimbriae provide the basic level of adhesion required to initiate biofilm formation, while the subsequent development of structural and protective mechanisms depends on other regulatory systems (Butsenko et al., 2020; Umitzhanov et al., 2023). The *fimA* gene was particularly stable, consistent with the conclusions of Dlamini et al. (2024), who identified its key role in initiating cell attachment. The frequent detection of this gene across all examined samples underscores its conservative nature and functional significance in ensuring the primary stage of bacterial adhesion to abiotic surfaces, such as equipment and transport belts. By contrast, Ćwiek et al. (2020) reported a more variable expression of *fimA*, while the results obtained in this study confirm its universality under poultry production conditions. This emphasizes its potential contribution to the resistance of bacterial populations against physical and chemical stressors at various stages of production. In addition, the high prevalence of *fimA* supports its consideration as a promising marker for molecular diagnostics and for monitoring the risk of product contamination.

The high detection rates of *csgD* and *bcsA* highlight the active involvement of biofilm formation mechanisms, which is consistent with the findings of Yuan et al. (2023) on the importance of these genes for bacterial survival in harsh environments. The strong expression of these genes indicates the ability of bacteria to actively form amyloid fibrils and a cellulose matrix, thereby creating robust three-dimensional structures that protect cells from external stresses. This is particularly significant in poultry production, where bacteria are exposed to various disinfectants and mechanical damage. However, Dai et al. (2021) reported low detection of *csgD* in poultry farms, which may be explained by differences in the research objects selected and the analytical methods employed. Such discrepancies may result from temporary fluctuations in *csgD* expression or the use of less sensitive molecular diagnostic techniques, underscoring the need to standardize approaches for assessing the biofilm potential of pathogens.

The *rpoS* gene exhibited the greatest variability, reflecting the heterogeneity of stress responses among isolates. Dallal et al. (2023) similarly observed that *rpoS* levels are strongly influenced by external conditions, a finding confirmed in this study. Unlike the conclusions of Kim et al. (2022), who described *rpoS* as having a secondary role in biofilm formation, the present data emphasize its importance for bacterial survival in production environments. The high variability in *rpoS* expression suggests significant differences among isolates in their ability to activate universal protective mechanisms in response to stressors such as disinfectants, temperature fluctuations, and mechanical forces along production lines. This supports the role of *rpoS* as a crucial regulator that enables pathogen adaptation to adverse conditions and enhances their persistence within the technological environment of poultry enterprises.

Expression analysis revealed higher transcriptional activity of the main biofilm formation genes in *S. typhimurium*. These findings are consistent with Musa et al. (2024), who also reported increased activation of biofilm pathways in this serotype. Elevated expression of key regulatory and structural genes, including *csgD*, *bcsA*, and *adrA*, in *S. typhimurium* confirms its capacity to intensively produce extracellular matrix and form stable biofilms. This provides the serotype with a significant advantage in industrial environments, where resistance to sanitation and survival under adverse conditions are critical. By contrast, Ramatla et al. (2024) reported predominant gene expression in *S. enteritidis*, a finding not corroborated in the present study, where *S. typhimurium* demonstrated greater molecular activity. These discrepancies may be attributed to geographical factors, differences in poultry management conditions, isolate characteristics, or analytical methods. Overall, the results highlight the importance of considering local factors when assessing the biofilm potential of different *Salmonella* serotypes.

Of particular interest is the high expression of *bcsA* and *adrA* in *S. typhimurium*, which is fully consistent with the conclusions of Abou Elez et al. (2021) and Hull et al. (2022) regarding the formation of dense and stable biofilm structures. These mechanisms provide this serotype with a clear advantage in poultry processing plants, where resistance to external influences is essential. The strong activity of *bcsA*, which encodes cellulose synthase, indicates the ability of bacteria to actively synthesize cellulose, thereby enhancing the strength and stability of the extracellular matrix. Increased expression of *adrA*, which regulates cGMP production, further reinforces this process by activating the relevant signaling pathways. This molecular organization grants *S. typhimurium* the ability to survive long-term on technological equipment and reduces the effectiveness of standard sanitary measures aimed at biofilm removal.

A phenotypic assessment of biofilm formation ability also revealed an advantage for *S. typhimurium*, confirming its high persistence potential. These results are consistent with the findings of Metaane et al. (2022), who emphasized the leading role of this serotype in the formation of persistent biofilms. In contrast, Chandra et al. (2023) reported the predominance of *S. enteritidis* under similar conditions. However, the larger sample size and broader coverage of technological stages in the present study make its results more robust and objective. The high proportion of strong biofilm formers among *S. typhimurium* isolates underscores the importance of monitoring and controlling this serotype in processing plants. Similar conclusions were drawn by Middlemiss et al. (2023), who highlighted the threat posed by stable biofilm formation. In contrast, Albicoro et al. (2024) argued that biofilms play only a minor role in the survival of *Salmonella* spp.; however, the present results refute this claim by demonstrating a direct link between biofilm activity and the likelihood of long-term bacterial persistence.

Cumulative data analysis confirms that biofilms play a vital role in the survival and dissemination of *Salmonella* spp. in poultry farms. Biofilm formation provides pathogens with significant protection against antiseptic agents, promotes their long-term persistence on abiotic surfaces, and increases the likelihood of horizontal transfer of antibiotic resistance genes (Zhusanbayeva et al., 2024; Boiko et al., 2025). Both high gene activity and strong phenotypic biofilm formation ability are particularly pronounced in *S. typhimurium*, necessitating strengthened control of this serotype at all stages of the production cycle. These biological features of *S. typhimurium* reflect its high epidemiological potential and risk of long-term persistence on equipment and within the environment of poultry processing plants. The findings parallel the conclusions of Brenner and Wang (2022), who stressed the need for targeted control of biofilms to minimize product contamination risks and ensure sanitary safety. They also emphasize the urgency of developing effective biofilm eradication methods and implementing innovative sanitation strategies.

Ultimately, this study confirms the critical role of slaughter lines in the spread of *Salmonella* spp. and reveals significant differences in biofilm potential between the serotypes. The high activity of *S. typhimurium* highlights the need for enhanced sanitary measures and the development of strategies to disrupt biofilms at all stages of the production cycle. The established patterns can serve as a foundation for optimizing monitoring systems and preventing bacterial contamination in the poultry industry.

CONCLUSIONS

In the course of the study conducted to identify serotype-specific molecular mechanisms regulating biofilm formation in *Salmonella* spp., substantial differences in the prevalence of serotypes were observed in poultry farms in Kazakhstan, Latvia, and Turkey at various stages of the technological process. A total of 238 bacterial strains were isolated from 340 samples, of which 52.1% belonged to *S. enteritidis* and 47.9% to *S. typhimurium*. The highest level of contamination was recorded on slaughter lines (43.3% of positive samples), confirming their critical role in the spread of pathogens. Country-

specific differences revealed the dominance of *S. enteritidis* in Kazakhstan (45.9% of isolates), which may be associated with poultry-keeping practices and the level of biosafety measures. In Latvia, *S. typhimurium* was predominant (64.7%), particularly on slaughter lines, indicating its adaptation and persistence in the production environment. In Turkey, the ratio of serotypes was approximately equal. Molecular analysis confirmed the high prevalence of genes responsible for biofilm formation. The highest detection rates were observed for *fimA* (94.1%) and *csgD* (88.7%), indicating a strong adhesive potential of the isolates. Expression analysis revealed significantly higher activity of key biofilm formation genes in *S. typhimurium*, particularly *csgD*, *bcsA*, and *adrA* ($p<0.01$), which correlated with its phenotypic ability to form denser and more stable biofilms. Phenotypic evaluation using crystal violet staining further confirmed the higher biofilm activity of *S. typhimurium*: the proportion of strong and very strong biofilm formers reached 65%, compared to 45% for *S. enteritidis*. This finding underscores the greater persistence and resistance potential of *S. typhimurium* in production environments.

The practical significance of this study for poultry production lies in the need to strengthen sanitary control at slaughter lines and to develop targeted strategies for biofilm eradication. Such measures are essential to reduce the persistence of *Salmonella* species in production environments and to improve overall food safety. By identifying the genetic factors contributing to biofilm formation, the study supports the development of more effective disinfection protocols and highlights the importance of prioritizing disinfectants capable of targeting these resilient biofilms. The inclusion of molecular monitoring of biofilm-associated genes in food safety control systems is recommended. A key limitation of the study was the absence of an assessment of seasonal factors and technological differences between enterprises, which warrants further in-depth analysis for a more comprehensive understanding of the epidemiological significance of the observed patterns. A promising direction for future research is the investigation of the effects of different disinfectants on the eradication of *Salmonella* spp. biofilms.

DECLARATIONS

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Data availability

The authors confirm that the data supporting the findings of this study are available in the article.

Authors' contribution

A. Zhusanbayeva, B. Biyashev, and Zh. Kirkimbaeva: conceptualization, methodology, data curation, writing-original draft preparation. B. Biyashev: visualization, investigation, and supervision. A. Zhylkaydar and G. Nurgozhaeva: software, validation, writing-reviewing, and editing. All authors read and approved the final manuscript.

Ethical considerations

All procedures performed in the study were in accordance with the ethical standards of the institutional research committee and with EU Directive 2010/63/EU for animal experiments.

Consent to publish

All authors agree to the publication of this manuscript.

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Competing interests

The authors declare no competing interests in this research and publication.

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