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The Basis for Variations in the Biofilm Formation by Different *Salmonella* Species and Subspecies: An In Vitro and In Silico Scoping Study

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Abstract: This study examined whether the presence/absence of biofilm-associated genes may indicate the potential for differences in the biofilm formation among the *Salmonella* species/subspecies. We conducted an in vitro study on the biofilm formation by eighteen *Salmonella* strains of different species/subspecies. Strains belonging to subspecies *enterica* were generally poorer biofilm formers than strains belonging to species *bongori* and subspecies *arizonae*, *diarizonae*, and *indica*. A broader in silico study was subsequently conducted. The presence/absence of 57 biofilm-associated genes was further investigated among 323 *Salmonella* whole genomes of various species/subspecies. The *lpfE* gene was present in in 88.2% of subspecies *enterica* but was absent in ~90.2–100% of other subspecies. The *sirA* gene was present in 11.8% of subspecies *enterica* and 2.9% of *S. diarizonae* genomes while absent in other species/subspecies. The *lpfe* gene and *sirA* gene in subspecies *enterica* negatively correlated with environmental biofilm formation. The *csrB* gene was present in 71.4% of the *S. arizonae* and 94.3% of *S. diarizonae* genomes but absent in other species/subspecies. The absence of *csrB* in subspecies *enterica* positively correlated with weaker environmental biofilm formation. This may contribute to subspecies *arizonae* and *diarizonae* being better biofilm formers.

Keywords: biofilm; genes; *Salmonella*; *enterica*; non-*enterica*

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1. Introduction

Salmonella is one of the leading causes of bacterial foodborne disease worldwide and also a common zoonotic pathogen. *S. enterica* and *S. bongori* are the two species of *Salmonella*. Most pathogenic *Salmonella* belong to the species *S. enterica*, which is traditionally further divided into subspecies *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) [1]. The subspecies *enterica* (I) has more than 2600 serovars [2]. More recently four more subspecies were identified, unnamed subsp VII and three novel subspecies A, B, and C [3]. The most common subspecies is *S. enterica* subspecies *enterica*, and this is generally isolated from humans and warm-blooded animals. Other subspecies and *S. bongori* are generally isolated from cold-blooded animals [4,5]. *Salmonella* has also been reported in environmental reservoirs, which have been seeded through contamination by animals and humans [6,7]. In most environments, it is difficult for planktonic *Salmonella* to survive due to a wide range of environmental stressors, which include desiccation, the scarcity of nutrients, and toxic compounds [8]. Biofilms represent one way in which *Salmonella* can ameliorate these stressors.

Biofilms are defined as a self-contained community of bacteria belonging to the same or different species that have attached to a biotic or abiotic surface and are enveloped by a layer of extracellular polymeric substances (EPS), which is composed of polysaccharides, proteins, lipids, and even DNA [9]. The benefit of a biofilm, especially in harsh conditions,

is that the EPS layer, which is produced by the bacteria within the biofilm, confers protection against most of the external threats mentioned previously whilst still allowing essential nutrients to enter and wastes to leave. Another benefit of a biofilm is that the cells within a biofilm, especially when there is a mixture of strains and species, are able to produce compounds, such as nutrients and enzymes that break down toxins, that help one another survive better [10]. It has also been observed that within the close confines of a biofilm, bacteria are able to transfer genes horizontally primarily through plasmids. These genes can contain anything from virulence factors, including biofilm-associated genes [11]. Virulence genes occur in *Salmonella* Pathogenicity Islands (SPI) located on chromosomes, plasmids, or bacteriophages [12]. SPIs are acquired by *Salmonella* during horizontal genetic transfer events, in which a large amount of DNA is transferred leading to the quick adaptation of *Salmonella* to stronger virulence. There are currently 24 SPIs identified, with most SPIs associated with the *enterica* species [12,13]. *S. bongori* has been reported to have a lower G + C content as compared to the *enterica* species and has three SPIs associated with it [5]. Subspecies *enterica* was separated from the other subspecies with the identification of SPI6. Little work has been conducted on SPIs with regards to the differences between the *enterica* and non-*enterica* subspecies [5]. Previous studies have also reported that plasmid types were associated with *Salmonella* serovars and the source of the isolates, but little is known with the non-*enterica* subspecies [14]. The ability of *Salmonella* to form biofilms on food processing surfaces, equipment, pipes, vegetables, and on food matrices can be of great concern, especially when the antimicrobials used are ineffective [8,15,16].

A large number of studies have been conducted on *S. enterica* subspecies *enterica* biofilm formation with a focus on *S.* Typhimurium and *S.* Enteritidis, as these two serovars are the primary causes of infections in humans [5,17,18]. A better understanding of biofilm formation by the entire *Salmonella* genus is important because there could be some exchange of genetic material among various species/subspecies impacting the biofilm formation. There has been very little work conducted on the relationship between the biofilm formation and the carriage of the relevant genes in *S. arizonae*, *S. diarizonae*, *S. houtenae*, *S. indica*, *S. salamae*, and *S. bongori* and their relationship to biofilm formation [19–21]. We conducted an in vitro study on biofilm formation by eighteen *Salmonella* strains of different species/subspecies. The subspecies *enterica* strains were generally poorer biofilm formers than species *bongori* and subspecies *arizonae*, *diarizonae*, and *indica*. A broader in silico study was subsequently conducted using 323 whole genomes. The presence/absence of 57 biofilm-associated genes was investigated to determine which species/subspecies had greater genetic potential to form biofilms.

2. Materials and Methods

2.1. In Vitro Study of Biofilm Formation by Selected Salmonella Strains

2.1.1. Bacterial Culture

Eighteen *Salmonella* strains isolated from previous studies [22,23] were used. These consisted of 10 isolates obtained from lizard stools, 7 isolates obtained from supermarket vegetables, and 1 isolate obtained from a bovine stool sample in Peninsular Malaysia. *S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076, obtained from the American Type Culture Collection (Manassas, VA, USA), were also used in this study. All strains were grown on xylose lysine deoxycholate (XLD; Oxoid, Basingstoke, UK) or in Tryptone Soy Broth (TSB; Oxoid, Basingstoke, UK) at 37 °C for 24 h under aerobic conditions. The subspecies of the *Salmonella* isolates were determined using a multiplex polymerase chain reaction (PCR) procedure as described by Lee et al. [24]

2.1.2. Biofilm Formation Assay

The biofilm assay was carried out as described previously by Mireles et al. [25] with modifications. The crystal violet staining method is a common method used to quantify the *Salmonella* biofilm formed [26,27]. It is based on the fact that crystal violet binds to the surface molecules of the bacteria and extracellular biofilm matrix. Bacterial cultures

were diluted in TSB to obtain an optical density of ~10^8 cfu/mL. Briefly, 200 µL of the bacterial culture was inoculated into a flat bottomed 96-well polystyrene microtiter plate (TPP®, Trasadingen, Switzerland). Uninoculated TSB was used as blanks. The microtiter plates were incubated under aerobic conditions for 18 h at 37 °C statically. We were interested in examining environmental biofilm formation, which is why the biofilms were grown under aerobic conditions, as described previously [28]. Biofilm formation under anaerobic, microaerophilic, or other conditions may be different. After incubation, broth cultures were poured out of the titer plates and the wells were gently rinsed twice with 200 µL of distilled water to remove the loosely attached bacteria. The plates were air dried for 20 min, and the wells were then stained using 200 µL of crystal violet for 15 min. The crystal violet was then removed, and the wells were rinsed twice again with 200 µL of distilled water and left to air dry for 20 min. The wells were then destained using 200 µL of 80:20 alcohol:acetone solution. The absorbance values of the plates were immediately read at 550 nm using Tecan Infinite® M200 (Tecan, Männedorf, Switzerland). The *Pseudomonas aeruginosa* strain MR2 isolated from the sewage treatment near Monash University Sunway Campus was used a control.

2.1.3. Screening of the Biofilm-Associated Genes

The experiment was repeated using PCR as described by Lee et al. [24] with slight modifications. Due to the significantly different annealing temperatures of the different primers used for the screening of biofilm-associated genes, monoplex PCRs were performed. The PCR reaction was carried out using a 25 µL reaction mixture which was composed of 14.4 µL of sterile distilled water, 5 µL 5× Green GoTaq Flexi buffer, 1.5 µL 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mixture, 1.25 µL of 0.1 mM forward and reverse primers, respectively, 0.125 µL of GoTaq® Flexi DNA polymerase, and 1.0 µL template genomic DNA. All the PCR procedures started with an initial denaturation step at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at the temperatures shown in Table 1 for 1 min, and extension at 72 °C for 6 min. There was a final extension at 72 °C for 10 min before a final hold at 4 °C. The positive controls used in this study were *S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076 for all genes except *tcfA*, since *tcfA* has been known to be *S.* Typhi specific. The amplicons were visualized on 2% agarose gel.

Table 1. Base sequence, expected amplicon size, and annealing temperature of the primers used for the monoplex virulence gene PCR and the gene function.

Gene	Sequence	Annealing Temperature (°C)	Size of Amplicons (bp)	Effect	Reference
lpfE	5′-TTTGATGCCAGCGTGTTTACTG-3′ 5′-AGTAGACCACCAGCAGAGGGAAAG-3′	50	525	Codes for long polar fimbriae that aid in the colonization of host intestinal wall	Bäumler et al. [29]
agfA	5′-TGCAAAGCGATGCCCGTAAATC-3′ 5′-TTAGCGTTCCACTGGTCGATGGTG-3′	61	151	Codes for curli adhesins that facilitate autoaggregation during biofilm formation	Bäumler et al. [29]
tcfA	5′-CATTTATTCTCAGGGGGAGCG-3′ 5′-CATCCTCTTTATCTGTTGCCACG-3′	51	1070	Typhi specific fimbriae required for virulence in humans	Townsend et al. [30]
bcfA	5′-TCCCCCGGGGATACTACAACCGTCACTGG-3′ 5′-GCGGATAAATCACCCTGGTC-3′	57	698	Codes for fimbriae involved in colonization of bovine gastrointestinal colonization	Townsend et al. [30]
pefA	5′-GGGAATTCTTGCTTCCATTATTGCACTGGG-3′ 5′-TCTGTCGACGGGGGATTATTTGTAAGCCACT-3′	58	526	Encodes for fimbriae necessary for adhesion to the murine gastrointestinal tract	Bäumler et al. [31]
bcsA	5′-GTCCCACATATCGTTACCGTCCTG-3′ 5′-CGCCGCATCATTTCTTCTCCC-3′	55	119	Plays a role in the production of extracellular cellulose required for biofilm formation	Barak et al. [32]
yshA	5′-CGGGATCCTTTTCTCTTGTATCGCCTTC-3′ 5′-CCCAAGCTTGAAGAAATACTTCGCCCCGA-3′	57	1000	Involved in the formation and secretion of extracellular polysaccharides required for biofilm formation. Especially under stress conditions.	Villareal et al. [33]

2.1.4. Expression of the Curli and Cellulose Assay

Colonies of all the bacterial isolates were analyzed on Luria Agar (LA; BD Difco, Sparks, MD, USA) without salt supplemented with Congo Red 40 µg/mL (Sigma Aldrich, St. Louis, MA, USA) as described by Malcova et al. [34] with some minor modifications. Briefly, each suspension was inoculated with each *Salmonella* isolate at ~10^8 cfu/mL. Serial decimal dilutions were conducted in phosphate-buffered saline (PBS; 2.7 mM KCl, 10 mM Na_2HPO_4, 17 mM KH_2PO_4, and 137 mM NaCl at pH 7.4; 1st Base, Singapore) and were inoculated onto the media. The inoculated media were then incubated for 24 h at 37 °C, followed by another 48 h at 24 °C. The different colony morphologies were recorded, and the respective expressions of fimbriae and cellulose genes were recorded as described by Bokranz et al. [35]. Briefly, a smooth and white (saw) morphology represented that neither curli fimbriae nor cellulose were expressed; a pink, dry, and red (pdar) morphology represented that only cellulose was expressed; brown, dry, and red (bdar) morphology represented that only curli fimbriae was expressed; and a red, dry, and rough (rdar) morphology represented both curli fimbriae and cellulose being expressed.

2.2. In silico Study of Biofilm-Associated Genes Across a Range of Salmonella Genomes

The presence of 57 biofilm-associated genes across 323 *Salmonella* genomes consisting of *S. enterica* subspecies *enterica* (*n* = 51), *S. enterica* subspecies *arizonae* (*n* = 70), *S. enterica* subspecies *diarizonae* (*n* = 70), *S. enterica* subspecies *indica* (*n* = 21), *S. enterica* subspecies *houtenae* (*n* = 51), *S. enterica* subspecies *salamae* (*n* = 52), and *S. bongori* (*n* = 8) were identified to determine whether the presence/absence of biofilm genes was linked to *Salmonella* subspecies and their ability to form biofilms. The 57 biofilm-associated genes were chosen from previous studies conducted mostly on *S. enterica* subspecies *enterica* [36,37]. The number of genomes per species/subspecies was selected based on the availability of the genomes on the National Centre for Biotechnology Information (NCBI) website and the information provided such as the year, location, and source of isolation. These strains were isolated from various sources and geographical locations globally from the year 1905 to 2022. The biofilm-associated genes were generally screened using a well characterized reference genome, *S*. Typhimurium strain LT2. The sequence of each reference gene was BLAST-searched against the 57 genomes respectively with a maximum e-value of 10^{-30} [38]. The reads of all these genomes were downloaded from the sequence read archive (SRA) in the NCBI and assembled using SPADES. The Quality Assessment Tool (QUAST) was used to determine the genome qualities of the final assemblies followed by annotation, using Rapid Annotations using Subsystems Technology (RAST) [39–41]. All the genomes were selected based on the availability of accession numbers and genome information as shown in the Supplementary Table S1. EvalG and EvalCon as described by Parks et al. [42] were used to determine the genome quality. The genomes were also annotated using the PROKKA annotation in order to determine the pan genome using the ROARY tool [43]. The pan genome was visualized by constructing a phylogenetic tree using the core genome sequences obtained from Roary. The phylogenetic tree was viewed and annotated using FigTree version 1.4.3, UK.

2.3. Statistical Analysis

All assays were conducted in triplicate. In the case of the biofilm formation assays, and viability assays, three independently grown bacterial broths were used. The data obtained were analyzed using SPSS 20 software (IBM Inc., Armonk, NY, USA). The data collected on biofilm formation by the respective strains were analyzed using a one-way analysis of variance (ANOVA) and a pairwise comparison of means that was performed using a post hoc Tukey test at 95% confidence level.

3. Results and Discussion

3.1. In Vitro Study of Biofilm Formation by Selected Salmonella Strains

The results of the biofilm assay using the microtiter plate method are shown in Figure 1. When the strains were arranged according to subspecies, a trend was observed where subspecies *enterica* appeared to be a weaker biofilm former than the other subspecies. Out of the ten strains under subspecies *enterica*, only ATCC 13076, M11, and M16 were at least half as good at forming biofilms as the *P. aeruginosa* control. Out of the remaining ten strains belonging to the other subspecies and *S. bongori*, all of them, except U3, produced at least half as much biofilm as *P. aeruginosa*, which was the control strain used in the study. *S. arizonae*, *S. diarizonae*, and *S. indica* were evidently better biofilm formers as compared to *S. bongori* in this study.

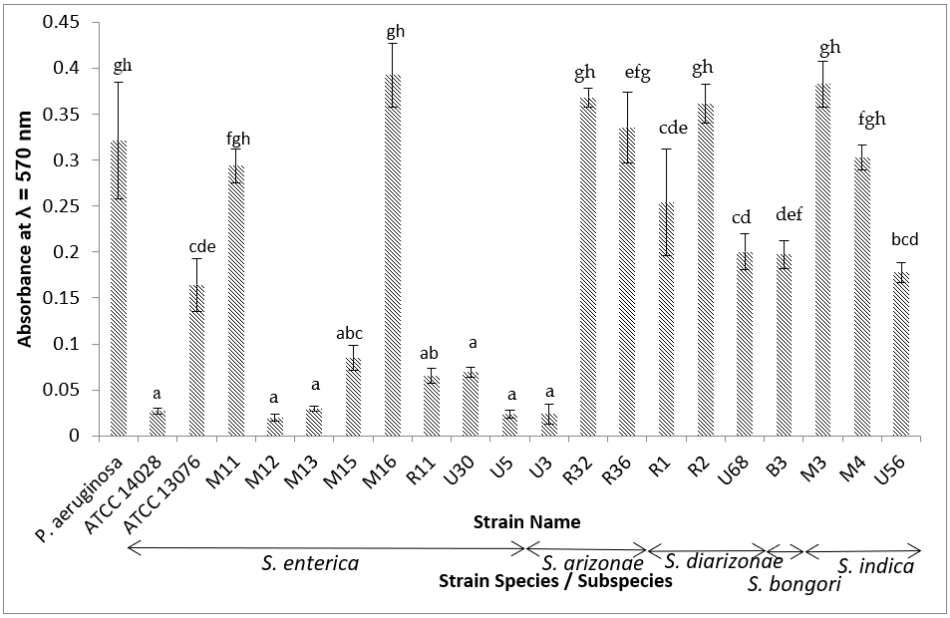

Figure 1. Biofilm formation on polystyrene surfaces by *Salmonella* strains. All results are presented as the mean ± SD where $n = 3$. The different letters represent significant differences in biofilm formation across the strains where $p < 0.05$.

Subspecies *enterica* tended to have more biofilm-associated genes with all ten enterica strains carrying between 57.1 and 85.7% of these. In comparison, the majority of the strains belonging to subspecies *arizonae*, *diarizonae*, and *indica* carried between 0 and 57.1% of the biofilm-associated genes. Subspecies *enterica* are better adapted at colonization and survival within the hosts as compared to the other subspecies [44]. Interestingly, the presence of several genes associated with biofilm formation in the genomes of the strains analyzed in this study may not influence the ability to form biofilms on polystyrene surfaces under conditions that simulate the environment. A limitation of our study was the number of analyzed strains of each subspecies. However, it can be observed that the higher percentage of biofilm-associated genes in subspecies *enterica* may be negatively correlated with biofilm formation in the environment, possibly making subspecies *enterica* a poorer biofilm former outside the host. Similarly, *S. bongori* carries a higher percentage of these genes as compared to subspecies *arizonae*, *diarizonae*, and *indica*, and it was a weaker biofilm former as compared to these three subspecies. Further studies can be carried out to analyze the role of these genes in the formation of biofilms by these *Salmonella* subspecies.

The expressions of curli and cellulose by the strains are presented in Table 2. Of the 20 strains screened, only two strains, *S. enterica* M11 and *S. arizonae* U3 expressed neither curli nor cellulose. The remaining 18 strains exhibited at least one of the two with seven of that 18 expressing both curli and cellulose. The formation of biofilms in *S. enterica* have been shown to rely heavily on the presence of curli fimbriae, which facilitates autoagglutination,

and cellulose, which forms the protective coating of a biofilm [29,32]. Previous studies have reported that the curli and cellulose production is variable in *S. enterica*, while certain strains of subspecies *arizonae* and *diarizonae* were not able to produce both cellulose and curli [5]. The expressions of both curli and cellulose in this study were at odds with the screening of the biofilm-associated genes as well with the ability to form biofilms. This shows that there could be other regulatory pathways that may be involved that are not present or active in these *Salmonella* strains. This is in agreement with previous studies that showed rdar, pdar, bdar, and saw morphotypes did not solely correlate with biofilm formation [45,46].

Table 2. Phylogenetic groups of *Salmonella* carrying biofilm-associated genes and its phenotypic traits on Congo Red agar.

Strain Name	Source	Subspecies/Species	Genes							Colony Morphology	Curli	Cellulose	Total Genes (%)
			lpfE	*agfA*	*bcsA*	*yshA*	*bcfA*	*pefA*	*tcfA*				
ATCC14028	-	*enterica*	+	+	+	+	+	+	-	rdar	√	√	85.7
ATCC13076	-	*enterica*	+	+	+	+	+	+	-	rdar	√	√	85.7
M11	Spinach	*enterica*	+	+	+	+	+	-	-	saw	×	×	71.4
M12	Spinach	*enterica*	+	+	+	+	+	-	-	pdar	×	√	71.4
M13	Spinach	*enterica*	+	+	+	+	+	-	-	pdar	×	√	71.4
M15	Cabbage	*enterica*	+	+	+	+	+	-	+	rdar	√	√	85.7
M16	Spinach	*enterica*	-	+	+	+	+	-	-	pdar	×	√	57.1
R11	Rural Lizard	*enterica*	-	+	+	+	+	-	+	pdar	×	√	71.4
U30	Urban Lizard	*enterica*	+	+	+	+	+	-	-	pdar	×	√	71.4
U5	Urban Lizard	*enterica*	-	+	+	-	+	+	-	rdar	√	√	57.1
R32	Rural Lizard	*arizonae*	-	+	+	-	+	+	-	pdar	×	√	57.1
R36	Rural Lizard	*arizonae*	-	+	+	-	+	-	-	rdar	√	√	42.9
U3	Urban Lizard	*arizonae*	-	+	+	-	+	-	-	saw	×	×	42.9
R1	Rural Lizard	*diarizonae*	-	+	+	-	+	-	-	pdar	×	√	42.9
R2	Rural Lizard	*diarizonae*	-	+	+	-	+	+	-	bdar	√	-	42.9
U68	Urban Lizard	*diarizonae*	-	+	+	-	+	-	-	rdar	√	√	42.9
B3	Cow	*S. bongori*	+	+	+	+	+	-	-	rdar	√	√	71.4
M3	Cabbage	*indica*	-	+	-	-	+	-	-	pdar	×	√	28.6
M4	Lettuce	*indica*	-	-	-	-	-	-	-	pdar	×	√	0.0
U56	Urban Lizard	indica	-	+	+	-	+	-	-	pdar	×	√	42.9

+ indicates that the *Salmonella* strain carries the biofilm-associated gene of concern, whilst – denotes that the *Salmonella* strain does not carry the biofilm gene of concern; √ exhibits the positive phenotype for a given trait, while × does not exhibit the phenotype for a given trait.

S. indica M4 was a strong biofilm former despite not possessing the *agfA*, *bcsA*, and *yshA* biofilm-associated genes investigated in this study, which are responsible for curli adhesin, cellulose production, and cellulose secretion, respectively, [29,32,33]. These three genes play a role in biofilm formation regardless of the surface being tested. Some bacteria have been shown to form biofilms without cellulose as the main component of the biofilm [47]. Another inconsistency that arose was the fact that *S. enterica* ATCC 14028, *S. enterica* M12, and *S. enterica* M13, which possessed all these three biofilm-associated genes, were poor biofilm formers. A possible reason for this is that mutations in the *bcs* operon, of which the *bcsA* gene is a part of, can adversely affect biofilm formation [48].

Based on the results shown in Figure 1, whilst each subspecies group had strong and weak biofilm formers, subspecies *enterica* was generally a weaker biofilm former as compared to the other groups. One possible explanation for the observed differences is that subspecies *arizonae*, *diarizonae*, *indica*, and *houtenae*, are generally found in cold-blooded animals as opposed to subspecies *enterica*, which is commonly found in warm-blooded animal hosts. We recognize that this study was limited with respect to both the number of strains and the conditions under which biofilm formation was investigated. It did, however, provide some interesting results, and we therefore undertook an in silico study to establish whether there was a broader basis for the results and to establish whether further research in the area is warranted.

3.2. In Silico Study of Biofilm-Associated Genes Across a Range of Salmonella Genomes

Across the 323 *Salmonella* genomes, the pan genome analysis indicated 1774 core genes present within ≥99% of *Salmonella* genomes. A phylogenetic tree was generated based on the entire pan genome as shown in Supplementary Figure S1, which consisted of a total of 60,259 genes. The genomes are listed according to the pan genome in Supplementary Table S1. The source of the *Salmonella* strains, presence/absence of biofilm-associated genes, and gene function are shown in Supplementary Table S1 and S2. The source of isolation was further categorized into clinical, nonclinical, and unclassified. Based on these categories, there were 166 clinical sources, 136 nonclinical sources, and 21 unclassified. To determine whether there was a more distinct relationship between the presence/absence of these biofilm-associated genes in each subspecies/species, we also categorized the source of isolation into fruit, herb, mammal, marsupial, reptile, meat, seafood, spice, and unclassified. Sometimes, other subspecies apart from *enterica* can be found in warm- blooded animals, cold-blooded animals, and humans, as seen in the screening of the 323 genomes. The numbers of each biofilm-associated gene present across species/subspecies are shown in Table 3. Among all the species/subspecies investigated, the percentage of all biofilm-associated genes carried by *S. enterica* and *S. arizonae* was 94.7% respectively, *S. diarizonae* was 96.5%, *S. houtenae* was 92.9%, *S. indica* was 87.7%, *S. salamae* was 89.5.%, and *S. bongori* was 82.46%. Within the *S. enterica* cohort, the presence/absence of genes was consistent across most of the genomes unlike the *S. arizonae* genomes.

The fimbriae (peF) are encoded by genes on the *Salmonella* plasmids [49]. These genes include *pefA* and *pefC*, which aid in the adhesion of *Salmonella* to surfaces such as epithelial cells [49,50]. Both *pefA* and *pefC* genes were reported in 15.7% of the subspecies *enterica* genomes, 1.4% of the subspecies *arizonae* genomes, and 5.8% of the subspecies *houtenae* genomes in this study. The presence of the *pefA* gene has been reported to be low in previous studies, with a recent study only reporting the presence of the *pefA* gene in 10.3% of the subspecies *enterica* strains investigated in their study [51–53]. Previous studies have shown that *S.* Enteritidis and *S.* Typhimurium plasmids carry the *pefA* and *pefC* genes [49]. Similarly, our in silico study showed that both the *pefA* gene and *pefC* gene were present across both these serovars. Both these genes were present across 60% of *S.* Enteritidis genomes and 40% of *S.* Typhimurium genomes, but our study also reported it across 50% of *S.* Bovismorbificans genomes among subspecies *enterica.* The *pefA* gene was also evident in both the *S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076 strains used in the in vitro study, which were weaker biofilm formers. The presence of *pefA* maybe negatively correlated with biofilm formation, and a possible reason for this could be that our study was investigating *Salmonella* biofilms in the environment rather than within a host. As such, there could be other regulatory pathways preventing it from being a strong biofilm former. The *pefA* gene and *pefC* gene were only present in 1.4% of the subspecies *arizonae* and 5.8% of the subspecies *houtenae* genomes, which could be associated with carrying the pef operon. However, with the screening of the in vitro study, both *S. arizonae* R32 and *S. diarizonae* R2, which carried the *pefA* gene, were strong biofilm formers. The presence of plasmids plays a role in recombination and virulence in *Salmonella*, and this could enhance its ability to form biofilms [54].

Table 3. Number of *Salmonella* strains carrying biofilm-associated genes.

Subspecies/ Species	*n*	Biofilm-Associated Gene																													
		bssR	*yciZ*	*ynfC*	*bssS*	*ygiK*	*gatC*	*wcaM*	*narG*	*fimF*	*fimG*	*fimH*	*csgA*	*csgB*	*csgE*	*cspA*	*bapA*	*csgD*	*cspE*	*adrA*	*trpE*	*ompR*	*rpoS*	*bcsC*	*bcsE*	*ygcB*	*sirA*	*hilA*	*hilC*	*barA*	*csrB*
S. enterica	51	■	■	■	■	■	34	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	42	6	■	■	■	□
S. arizonae	70	59	8	55	60	58	3	53	56	34	□	66	59	62	30	50	59	59	60	66	64	53	53	55	53	57	□	57	53	58	50
S. diarizonae	70	■	■	■	■	■	2	■	■	■	1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	2	■	■	■	66
S. houtenae	51	■	■	■	■	■	4	50	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	5	□	■	■	■	□
S. indica	21	■	■	■	■	■	1	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	□
S. salamae	52	■	■	■	■	■	30	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	24	□	■	■	■	□
S. bongori	8	■	■	■	■	■	□	■	■	□	□	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■	□

Subspecies/Species	*n*	Biofilm-associated gene																													
		yjcC	*fabR*	*flgK*	*rfbA*	*nusB*	*waaG*	*rfaB*	*pmp*	*waaL*	*waaJ*	*waaK*	*waaB*	*waaO*	*bcsA*	*bcsB*	*bcsZ*	*lpfE*	*csgC*	*yshA*	*luxS*	*csrA*	*sdiA*	*bdcA*	*pefA*	*pefC*	*marT*	*pagC*
S. enterica	51	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	45	■	■	■	■	■	■	8	8	■	■
S. arizonae	70	65	58	55	60	55	56	64	56	□	57	5	64	62	59	59	55	3	55	51	57	54	57	3	1	1	4	67
S. diarizonae	70	69	■	■	■	■	■	■	■	1	■	69	■	■	■	■	■	3	69	■	■	■	■	3	□	□	66	■
S. houtenae	51	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	5	■	■	■	■	■	■	3	3	5	■
S. indica	21	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	□	■	■	■	■	■	■	□	□	■	■
S. salamae	52	■	■	■	■	■	33	■	51	□	■	■	■	■	■	■	■	1	51	50	51	51	51	48	□	□	49	■
S. bongori	8	■	■	■	■	■	■	■	■	□	□	■	■	■	■	■	■	■	■	■	■	■	■	■	□	□	■	■

■ means that all the genomes of the particular subspecies/species carry the biofilm gene of concern. □ means all genomes of the particular subspecies/species do not carry the biofilm gene of concern. The numbers corresponding to each gene represent numbers of genomes within a subspecies/species carrying the biofilm gene of concern.

The *lpfE* gene, which codes for long polar fimbriae, aids in the colonization of the host intestinal wall [29]. Previous studies have reported that the plasmid encoded phase 2 flagellar regions carrying both *lpf* (long polar fimbriae) and *pef* (plasmid encoded fimbriae) genes, commonly associated with human salmonellosis were absent in *S.* Sofia [5,55]. This correlates with the results of this study, which showed that all the subspecies *salamae* genomes, which were characterized to *S.* Sofia lacked the *lpfe*, *pefA*, and *pefC* genes. All the *S. salamae* genomes irrespective of serovar and the source of isolation investigated in this study were reported to lack the *pefA* and *pefC* genes, while the *lpfE* gene was only evident in 1.9% of the *S. salamae* genomes. The *lpfE* gene was present in 70% of *S. enterica* and in 100% of *S. bongori* strains, while it was absent across the remaining strains in the in vitro study. This correlates with the broader in silico investigation, as 88.2% of the *S. enterica* genomes and 100% of the *S. bongori* genomes carried the *lpfe* gene. The *lpfE* gene was absent in 100% of the *S. indica* genomes but was present in 4.3% of the *S. arizonae*, 4.3% of the *S. diarizonae*, 9.8% of the *S. houtenae*, and 1.9% of the *S. salamae* genomes. The *lpfE* gene was only present in the *S.* salamae genome isolated from a reptile. In the case of *S. arizonae*, *lpfE* was only present in strains isolated from animal-based sources, while for *S. diarizonae*, the *lpfE* gene was only present in strains isolated from food or humans. This was not the case when compared with subspecies *enterica*, where 88.2% of subspecie *enterica* genomes carried this gene irrespective of the source. The *lpfE* gene was present in 88.2% of subspecies *enterica* but was absent in ~90.2–98.1% of the other subspecies. The relatively high presence of the *lpfE* gene in subspecies *enterica* is correlated with previous studies that showed that the lpf operon has been associated with subspecies *enterica* [56]. Although subspecies *enterica* has a high occurrence of the *lpfE* gene, which aids in the colonization of the host intestinal wall, it was a weaker biofilm former in our study. The presence of this gene may be negatively correlated with the biofilm formation of subspecies *enterica* in the environment rather than in the host.

Previous studies have determined the expression of cellulose using morphotypic screening to determine the expression of extracellular cellulose and curli fimbriae using Congo Red impregnated onto the culture media to enable the differentiation of the bacterial gene expression based on the colony morphology, which prompted us to use this methodology [34]. The rdar morphotype is caused by the co-expression of the curli fimbriae and cellulose associated genes, bdar is associated with the curli fimbriae synthesis genes, saw is associated with the cellulose synthesis genes, and pdar is associated with the presence of cellulose production but the absence of fimbriae biosynthesis genes [48,57–59]. As mentioned earlier, the *bcsA* gene is essential for cellulose secretion and was only absent in 66.7% of the *S. indica* strains in the in vitro study. This was not the same with the broader in silico investigation, as this gene was present across all species and subspecies. The presence/absence of this *bcsA* gene was only variable across the *S. arizonae* genomes investigated in the in silico study with regard to biofilm formation. The *adrA* gene is essential in enhancing the cellulose synthesis to enhance biofilm formation. In the in silico study, all species and subspecies carried this gene with the exception of 5.7% of the subspecies *arizonae* genomes. Bacterial cells exhibiting the rdar and pdar morphotypes are related to biofilm formation and are closely related to the *adrA* gene. However, in the in vitro study, only 10% of subspecies *enterica*, 33% of subspecies *arizonae*, and subspecies *diarizonae*, respectively, did not express the cellulose morphotype. This may suggest that most *Salmonella* genomes irrespective of subspecies may carry this gene with some exceptions. The other essential gene involved in exhibiting the rdar morphotype is *csgD*, as both *adrA* and *csgD* are involved in co-expressing cellulose and curli fimbriae [46]. There were eleven *S. arizonae* genomes from mostly clinical sources lacking *csgD*. Some of these *S. arizonae* genomes were also lacking the *bcsC* involved in cellulose production. This is consistent with our in vitro study, as two strains of *S. arizonae* did not express the curli fimbriae. This could be attributed to the fact that they might not be carrying *csgD*.

The sigma factor, *rpoS*, regulates the expression of ~100 genes associated with environmental stressors, while the *hilA* gene is a transcriptional activator involved in *Salmonella*

invasion associated with the transcription of genes in SPI1 [36,60]. Both *rpoS* and *hilA*, which is essential in the biofilm formation of *S.* Typhimurium, were observed to be present across all species and subspecies with variation observed among the *S. arizonae* genomes [36]. The *luxS* gene is involved in the molecular or *Salmonella* communication signals associated with biofilm formation [61]. The *luxS* gene, mostly evident in *S. enterica* genomes, was also observed to be present across most species/ subspecies with the exception of some *S. arizonae* genomes and one *S. salamae* genome. The serovar of these *Salmonella* genomes may have contributed to the absence of the *luxS* in the *S. salamae* genome, as the other *S. salamae* genomes with a known serovar such as Sofia were observed to carry this gene.

The *bssR* gene, which is essential in cell signaling [62] was present across all subspecies investigated in this study with some variation across the *S. arizonae* genomes. The *bssR* gene involved in biofilm regulation has been previously reported in *S. enterica*, and its expression was downregulated in *S.* Typhi biofilm [63]. However, this same gene was significantly upregulated in *Escherichia coli* biofilms, which is very closely related to *Salmonella* [63]. The *yciZ* and *ynfC* genes are reported to co-occur with *bssR*. To our knowledge, there has been no work conducted with *yciZ* in relation to the biofilm formation of *Salmonella.* Approximately 88.6% of the *S. arizonae* genomes lacking this gene clustered together in the pan genome irrespective of the source of isolation. The *yciZ* gene was evident in ~98.6–100% of the genomes of other subspecies and species, which may help with its biofilm formation. The *gatC* gene, a gene regulator for biofilm formation has been reported among antimicrobial resistant *S. enterica* subspecies *enterica* isolated from poultry [64]. Among the subspecies *enterica* genomes investigated in this study, 66.7% of the genomes carried the *gatC* gene. This gene was absent in all the *S.* Dublin and *S.* Enteritidis genomes, 16.7% of the *S.* Bovismorbificans genomes, and 40% of the *S.* Saintpaul and *S.* Newport genomes, among the subspecies *enterica*. However, in the non-*enterica* subspecies investigated in our study, *gatC* was present in 57.7% of the subspecies *salamae*, 7.8% of the subspecies *houtenae*, 4.8% of the subspecies *indica*, 4.3% of the subspecies *arizonae*, and 2.8% of the subspecies *diarizonae*. The *gatC* gene has been implicated to be associated with invasive isolates in a host [65]. Thus, the presence of this gene in subspecies *enterica* may be negatively correlated with strong biofilm formation in the environment.

The *fimF*, *fimG*, and *fimH* genes are responsible for the adhesive capability and for the initial contact of biofilm formation on host cells [66,67]. The *fimF* and *fimH* associated with type 1 fimbriae in *S.* Typhimurium were reported across all subspecies in this study. These genes were only absent across all *S. bongori* genomes. Some of the genes investigated in this study could be *Salmonella* species/subspecies/serovar determinant genes, which could contribute towards better biofilm formation. To our knowledge, these genes have yet to be reported in *S. bongori*, as there is very little work on it. Previous studies have reported that certain strains of *S.* Typhimurium carrying *fimF* were good biofilm formers, while *fimH* is important for the initial attachment on epithelial cells [68]. The *fimG* gene associated with Type 1 fimbriae is essential for the initial attachment and has been reported to enhance the biofilm formation in *E. coli* [69]. This was shown with a mutant of *fimG* that decreased the biofilm formation of *E. coli*. This gene has rarely been reported among subspecies *enterica* and has not been reported in *S.* Typhimurium [70]. This agrees with our study, as all subspecies *enterica* lacked this gene. In addition, this gene was also absent across all other subspecies and species with the exception of one genome of *S. diarizonae*.

The gene *sirA* is important in enhancing biofilm formation by increasing the expression of type 1 fimbriae and is essential in activating the *csrB* for biofilm formation. This gene has been reported to be associated with *Salmonella* host cell invasion [71]. It was apparent in our in silico study that this gene was only present in 11.8% of subspecies *enterica* and 2.8% of subspecies *diarizonae* genomes. It is interesting to note that *S.* Dublin and *S.* Newport were reported to carry the *sirA* gene among subspecies *enterica* in this study. The presence of this gene in subspecies *enterica* was negatively correlated with the biofilm formation of *Salmonella* in the environment. None of the subspecies *enterica* genomes carried the *csrB* gene. The absence of the *csrB* gene in subspecies *enterica* may contribute to this

subspecies being a poorer biofilm former. The presence of the *csrB* gene was evident in 71.4% of subspecies *arizonae* and 94.3% of subspecies *diarizonae* genomes. All the remaining subspecies lacked *csrB*. The presence of the *csrB* gene in both subspecies *arizonae* and *diarizonae* was positively correlated with biofilm formation and may contribute to these subspecies having better potential in biofilm formation as compared to the other subspecies and species.

The *ygcB* gene, which is involved in the CRISPR-associated helicase/endonuclease *Cas3*, is associated with the regulation of *S. enterica* biofilm formation. This gene was absent across 60% of S. Newport and 100% of *S.* Bovismorbificans genomes among the subspecies *enterica* [72]. This included *S.* Newport 2393, which lacked all *cas* genes, and this was related to its stress response to heat in the presence of iron [73]. The presence/absence of this gene in subspecies enterica could be serovar specific [73]. This gene was also absent in 90.2% of the *S. houtenae* genomes clustering together in the pan genome.

An important factor that can contribute to enhance the virulence of *Salmonella* is the occurrence of recombination and the presence of novel genes which may impact the diversity of the lipopolysaccharide antigenic factor [54,74]. These changes can also contribute to the presence of lipopolysaccharides-associated genes such as *waaL*, *waaJ*, *waaK*, *waaB*, and *waaO*, which are essential in biofilm formation. The *waaL*, *waaJ*, and *waaB* genes are generally conserved among *S.* Typhimurium [75]. However, in this study, only *waaJ* and *waaB* were present across all subspecies *enterica* genomes irrespective of serovar, while *waal* was absent across all subspecies *enterica* genomes irrespective of serovar. The gene *waaL* was only evident in 1.4% of the *S. diarizonae* genomes investigated in this study. Previous studies reported that the deletion of the *waaJ* gene in subspecies *enterica* resulted in a lower amount of lipopolysaccharide being produced than the deletion of the *waaL* gene in *S. enterica* [76]. In addition, the deletion of the *waaJ* gene in subspecies *enterica* also exhibited a decrease in resistance to ultraviolet-, acid-, and alkaline-based treatments [76]. This shows that the presence of this gene is essential to ensure the integrity of the lipopolysaccharide layer to form better biofilms as well as to resist intervention strategies using ultraviolet-, acid-, and alkaline-based treatments. In our study, the *waaJ* gene was absent across all *S. bongori* genomes and was present across all genomes of *S. diarizonae*, *S. houtenae*, *S. indica*, and *S.* salamae. Similar to most other genes in *S. arizonae*, there was a variation in the presence/absence of *waaJ*. The *pagC* has been shown to influence lipopolysaccharide production in *S. enterica* [77]. In our study, the *pagC* gene was present across most genomes irrespective of subspecies. The *bdca* gene aids in biofilm dispersal in *E. coli* by interacting with the intracellular signal cyclic diguanylate (c-di GMP) when there is a reduced concentration of c-di GMP [78]. This leads to an increase in extracellular DNA production and a lower amount of extracellular polysaccharide production (EPS) and cellular aggregation, finally leading to the biofilm dispersal [31]. In this study, we noted that *bdcA* was only present across all the genomes of *S. enterica*, *S. houtenae*, *S. indica*, and *S. bongori*. To our knowledge, the concentration of c-di GMP has yet to be investigated in detail with regard to *Salmonella*, which may lead to uncertainties about whether this gene may enhance or decrease biofilm formation.

The *marT* gene is a regulator in *S.* Typhimurium biofilms [68]. The presence of this gene is essential in regulating the expression of 14 other genes including *csgA* and *csgD* to enhance biofilm formation. This gene was present in 100% of subspecies *enterica*, *indica* and *S. bongori*, 94.4% of subspecies *diarizonae*, 94.2% of subspecies *salamae*, 9.8% of subspecies *houtenae*, and 5.7% of subspecies *arizonae* genomes. As *marT* is only present in a number of *S. houtenae* and *S. arizonae* genomes, this may impact the expression of *csgA* and *csgD* to enhance biofilm formation. Although the *marT* gene was present across all subspecies *enterica* genomes, it may not be expressed or might not be regulating both the *csgA* and *csgD* genes to enhance biofilm formation, making this subspecies a poorer biofilm former.

Some of the genes screened in this study have been investigated in the human host in previous studies instead of in the environment for biofilm formation. As such, the presence of some genes may be negatively correlated with biofilm formation in some subspecies

as mentioned above. All the genomes and strains investigated in our in vitro and in silico study, respectively, were characterized to the species and subspecies level. However, there were only two strains characterized to the serovar level in the in vitro study. These two strains were *S.* Typhimurium ATCC 14028 and *S.* Enteritidis ATCC 13076, which were also used as positive controls for the screening of the biofilm-associated genes. With regard to the genomes investigated in the in silico study, all the subspecies *enterica* genomes and some of the other subspecies genomes were characterized to the serovar level. Future studies should more deeply characterize all genomes and strains to the serovar level to have a better understanding of biofilm formation.

4. Conclusions

Our in vitro study on biofilm formation showed that subspecies *enterica* were generally poorer biofilm formers than strains belonging to the species *bongori* and subspecies *arizonae*, *diarizonae*, and *indica.* The broader in silico study investigating *Salmonella* whole genomes of all species and subspecies supported our in vitro study. It showed that the *lpfE* gene was present in 88.2% of subspecies *enterica* genomes but was absent in ~90.2–100% of other subspecies genomes. The *sirA* gene was present in 11.8% of subspecies *enterica* and 2.8% of subspecies *arizonae* genomes but absent in other species and subspecies. The presence of the *lpfE* gene and *sirA* gene in subspecies *enterica* may be negatively correlated with *Salmonella* biofilm formation in the environment. The *csrB* gene was present in 71.4% of the *S. arizonae* and 94.3% of *S. diarizonae* genomes but absent in other species/subspecies. The absence of *csrB* in subspecies *enterica* may be positively correlated with weaker biofilm formation in the environment. These differences among *Salmonella* species/subspecies contribute to the potential for differences in environmental biofilm formation, possibly making subspecies *enterica* a poorer biofilm former as compared to the other species and subspecies. Further studies should focus on in vitro studies of biofilm formation across all species and subspecies from various sources worldwide. Gene expression studies utilizing whole transcriptome sequencing should also be conducted to complement the in vitro studies of *Salmonella* biofilm formation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol3030058/s1. Figure S1: Phylogenetic tree of core genome sequences of Salmonella genomes (n = 323). The maximum-likelihood method was used to generate the tree across the core genomes. Table S1: Pan genome; Table S2: Gene Function.

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Data Availability Statement: All publicly available whole genome sequence of *Salmonella* strains have been deposited at the PATRIC database listed in Supplementary Table S1. Supplementary Figure S1: Phylogenetic tree of core genome sequences of Salmonella genomes (n = 323).

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References

1. Grimont, P.A.; Weill, F.X. Antigenic formulae of the *Salmonella* serovars. In *WHO Collaborating Centre for Reference and Research on Salmonella*; Institut Pasteur: Paris, France, 2007; Volume 9, pp. 1–66.
2. Gong, J.; Zhuang, L.; Zhu, C.; Shi, S.; Zhang, D.; Zhang, L.; Yu, Y.; Dou, X.; Xu, B.; Wang, C. Loop-mediated isothermal amplification of the *sefA* gene for rapid detection of *Salmonella* Enteritidis and *Salmonella* Gallinarum in chickens. *Foodborne Pathog. Dis.* **2016**, *13*, 177–181. [CrossRef] [PubMed]
3. Alikhan, N.F.; Zhou, Z.; Sergeant, M.J.; Achtman, M. A genomic overview of the population structure of *Salmonella*. *PLoS Genet.* **2018**, *14*, e1007261. [CrossRef]
4. Eng, S.K.; Pusparajah, P.; Ab Mutalib, N.S.; Ser, H.L.; Chan, K.G.; Lee, L.H. *Salmonella*: A review on pathogenesis, epidemiology and antibiotic resistance. *Front. Life Sci.* **2015**, *8*, 284–293.

5. Lamas, A.; Miranda, J.M.; Regal, P.; Vázquez, B.; Franco, C.M.; Cepeda, A.A. Comprehensive review of non-enterica subspecies of *Salmonella enterica*. *Microbiol. Res.* **2018**, *206*, 60–73. [CrossRef] [PubMed]
6. Liu, H.; Whitehouse, C.A.; Li, B. Presence and persistence of *Salmonella* in water: The impact on microbial quality of water and food safety. *Front. Public Health* **2018**, *6*, 159. [CrossRef] [PubMed]
7. Underthun, K.; De, J.; Gutierrez, A.; Silverberg, R.; Schneider, K.R. Survival of *Salmonella* and *Escherichia coli* in two different soil types at various moisture levels and temperatures. *J. Food Prot.* **2018**, *81*, 150–157. [CrossRef]
8. Cadena, M.; Kelman, T.; Marco, M.L.; Pitesky, M. Understanding antimicrobial resistance (AMR) profiles of *Salmonella* biofilm and planktonic bacteria challenged with disinfectants commonly used during poultry processing. *Foods* **2019**, *8*, 275. [CrossRef]
9. Costerton, J.W.; Lewandowski, Z.; Caldwell, D.E.; Korber, D.R.; Lippin-Scott, H.M. Microbial Biofilms. *Annu. Rev. Microbiol.* **1995**, *49*, 711–745. [CrossRef]
10. Nadell, C.D.; Xavier, J.B.; Foster, K.R. The sociobiology of biofilms. *FEMS Microbiol. Rev.* **2008**, *33*, 206–224. [CrossRef]
11. Ghigo, J.M. Natural conjugative plasmids induce bacterial biofilm development. *Nature* **2001**, *412*, 442–445. [CrossRef]
12. Liu, X.; Jiang, Z.; Liu, Z.; Li, D.; Liu, Z.; Dong, X.; Yan, S. Research Progress of *Salmonella* Pathogenicity Island. *Int. J. Biol. Sci.* **2023**, *2*, 7–11. [CrossRef]
13. Kombade, S.; Kaur, N. Pathogenicity island in *Salmonella*. In *Salmonella spp.—A Global Challenge*; IntechOpen: London, UK, 2021.
14. Zhao, S.; Li, C.; Hsu, C.H.; Tyson, G.H.; Strain, E.; Tate, H.; Tran, T.T.; Abbott, J.; McDermott, P.F. Comparative genomic analysis of 450 strains of *Salmonella enterica* isolated from diseased animals. *Genes* **2020**, *11*, 1025. [CrossRef] [PubMed]
15. Sarjit, A.; Dykes, G.A. Antimicrobial activity of trisodium phosphate and sodium hypochlorite against *Salmonella* biofilms on abiotic surfaces with and without soiling with chicken juice. *Food Control* **2017**, *73*, 1016–1022. [CrossRef]
16. Shastry, R.P.; Ghate, S.D.; Banerjee, S. Culture dependent and independent detection of multiple extended beta-lactamase producing and biofilm forming *Salmonella* species from leafy vegetables. *Biocatal. Agric Biotechnol.* **2021**, *38*, 102202. [CrossRef]
17. European Food Safety Authority and European Centre for Disease Prevention and Control (ECDC). The European Union summary report on trends and sources of zoonoses, zoonotic agents and foodborne outbreaks in 2015. *EFSA J.* **2016**, *14*, 4634.
18. Simm, R.; Ahmad, I.; Rhen, M.; Le Guyon, S.; Römling, U. Regulation of biofilm formation in *Salmonella enterica* serovar Typhimurium. *Future Microbiol.* **2014**, *9*, 1261–1282. [CrossRef]
19. Desai, P.T.; Porwollik, S.; Long, F.; Cheng, P.; Wollam, A.; Clifton, S.W.; Weinstock, G.M.; McClelland, M. Evolutionary genomics of *Salmonella enterica* subspecies. *MBio* **2013**, *4*, e00579-12. [CrossRef] [PubMed]
20. Sathyabama, S.; Kaur, G.; Arora, A.; Verma, S.; Mubin, N.; Mayilraj, S.; Agrewala, J.N. Genome sequencing, annotation and analysis of *Salmonella enterica* sub species *salamae* strain DMA-1. *Gut Pathog.* **2014**, *6*, 8. [CrossRef]
21. Wang, C.X.; Zhu, S.L.; Wang, X.Y.; Feng, Y.; Li, B.; Li, Y.G.; Johnston, R.N.; Liu, G.R.; Zhou, J.; Liu, S.L. Complete genome sequence of *Salmonella enterica* subspecies *arizonae* str. RKS2983. *Stand. Genom. Sci.* **2015**, *10*, 30. [CrossRef]
22. Cheng, B.; Wong, S.; Dykes, G. *Salmonella* associated with captive and wild lizards in Malaysia. *Herpetol. Notes* **2014**, *7*, 145–147.
23. Perera, A.; Clarke, C.M.; Dykes, G.A.; Fegan, N. Characterization of Shiga toxigenic *Escherichia coli* O157 and Non-O157 isolates from ruminant feces in Malaysia. *Biomed. Res. Int.* **2015**, *11*, 2015.
24. Lee, K.; Iwata, T.; Shimizu, M.; Nakadai, A.; Hirota, Y.; Hayashidani, H. A novel multiplex PCR assay for *Salmonella* subspecies identification. *J. Appl. Microbiol.* **2009**, *107*, 805–811. [CrossRef]
25. Mireles, J.R.; Toguchi, A.; Harshey, R.M. *Salmonella enterica* serovar Typhimurium swarming mutants with altered biofilm-forming abilities: Surfactin inhibits biofilm formation. *J. Bacteriol.* **2001**, *183*, 5848–5854. [CrossRef]
26. Chen, S.; Feng, Z.; Sun, H.; Zhang, R.; Qin, T.; Peng, D. Biofilm-formation-related Genes csgD and bcsA promote the vertical transmission of *Salmonella* Enteritidis in chicken. *Front. Vet. Sci.* **2021**, *7*, 625049. [CrossRef] [PubMed]
27. Obe, T.; Nannapaneni, R.; Schilling, W.; Zhang, L.; Kiess, A. Antimicrobial tolerance, biofilm formation, and molecular characterization of *Salmonella* isolates from poultry processing equipment. *J. Appl. Poult. Res.* **2021**, *30*, 100195. [CrossRef]
28. Stepanović, S.; Ćirković, I.; Ranin, L.; Svabić-Vlahović, M. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett. Appl. Microbiol.* **2004**, *38*, 428–432. [CrossRef]
29. Baumler, A.J.; Gilde, A.J.; Tsolis, R.M.; van der Velden, A.W.; Ahmer, B.M.M.; Heffron, F. Contribution of horizontal gene transfer and deletion events to development of distinctive patterns of fimbrial operons during evolution of *Salmonella* serotypes. *J. Bacteriol.* **1997**, *179*, 317–322. [CrossRef]
30. Townsend, S.M.; Kramer, N.E.; Edwards, R.; Baker, S.; Hamlin, N.; Simmonds, M.; Stevens, K.; Maloy, S.; Parkhill, J.; Dougan, G.; et al. *Salmonela enterica* serovar Typhi possess a unique repertoire of fimbrial gene sequences. *Infect. Immun.* **2001**, *69*, 2891–2901. [CrossRef]
31. Bäumler, A.J.; Tsolis, R.M.; Bowe, F.A.; Kusters, J.G.; Hoffman, S.; Heffron, F. The *pef* fimbrial operon of *Salmonella* Typhimurium mediates adhesion to murine small intestine and is necessary for fluid accumulation in the infant mouse. *Infect. Immun.* **1996**, *64*, 61–68. [CrossRef]
32. Barak, J.D.; Jahn, C.E.; Gibson, D.L.; Charkowski, A.O. The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. *Mol. Plant Microbe Interact.* **2007**, *20*, 1083–1091. [CrossRef]
33. Villareal, J.M.; Hernandez-Lucas, I.; Gil, F.; Calderon, I.L.; Calva, E.; Saavedra, C.P. cAMP receptor protein (CRP) positively regulates the *yihU-yshA* operon in *Salmonella enterica* serovar Typhi. *Microbiology* **2011**, *157*, 636–647. [CrossRef] [PubMed]

34. Malcova, M.; Hradecka, H.; Karpiskova, R.; Rychlik, I. Biofilm formation in field strains of *Salmonella enterica* serovar Typhimurium: Identification of a new colony morphology type and the role of SGI1 in biofilm formation. *Vet. Microbiol.* **2008**, *129*, 360–366. [CrossRef] [PubMed]
35. Bokranz, W.; Wang, X.; Tschäpe, H.; Römling, U. Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. *J. Med. Microbiol.* **2005**, *54*, 1171–1182. [CrossRef]
36. Roy, P.K.; Song, M.G.; Park, S.Y. Impact of quercetin against *Salmonella* Typhimurium biofilm formation on food–contact surfaces and molecular mechanism pattern. *Foods* **2022**, *11*, 977. [CrossRef] [PubMed]
37. Yuan, L.; Liu, Y.; Fan, L.; Chen, C.; Yang, Z.; Jiao, X.A. Biofilm formation, antibiotic resistance, and genome sequencing of a unique isolate *Salmonella* Typhimurium M3. *Qual. Assur. Saf. Crops* **2023**, *15*, 114–122. [CrossRef]
38. Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* **1997**, *25*, 3389–3402. [CrossRef]
39. Bankevich, A.; Nurk, S.; Antipov, D.; Gurevich, A.A.; Dvorkin, M.; Kulikov, A.S.; Lesin, V.M.; Nikolenko, S.I.; Pham, S.; Prjibelski, A.D.; et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* **2012**, *19*, 455–477. [CrossRef]
40. Brettin, T.; Davis, J.J.; Disz, T.; Edwards, R.A.; Gerdes, S.; Olsen, G.J.; Olsen, R.; Overbeek, R.; Parello, B.; Pusch, G.D.; et al. RASTtk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. *Sci. Rep.* **2015**, *5*, 8365. [CrossRef]
41. Klimke, W.; Agarwala, R.; Badretdin, A.; Chetvernin, S.; Ciufo, S.; Fedorov, B.; Kiryutin, B.; O'Neill, K.; Resch, W.; Resenchuk, S.; et al. The national center for biotechnology information's protein clusters database. *Nucleic Acids Res.* **2009**, *37*, D216–D223. [CrossRef]
42. Parks, D.H.; Imelfort, M.; Skennerton, C.T.; Hugenholtz, P.; Tyson, G.W. Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res.* **2014**, *25*, 1043–1055. [CrossRef]
43. Page, A.J.; Cummins, C.A.; Hunt, M.; Wong, V.K.; Reuter, S.; Holden, M.T.G.; Fookes, M.; Falush, D.; Keane, J.A.; Parkhill, J. Roary: Rapid large-scale prokaryote pan genome analysis. *Bioinformatics* **2015**, *31*, 3691–3693. [CrossRef] [PubMed]
44. Baumler, A.J.; Tsolis, R.M.; Ficht, T.A.; Adams, L.G. Evolution of host adaptation in *Salmonella enterica*. *Infect. Immun.* **1998**, *66*, 4579–4587. [CrossRef] [PubMed]
45. Avila-Novoa, M.G.; Guerrero-Medina, P.J.; Navarrete-Sahagún, V.; Gómez-Olmos, I.; Velázquez-Suárez, N.Y.; De la Cruz-Color, L.; Gutiérrez-Lomelí, M. Biofilm formation by multidrug-resistant serotypes of *Salmonella* isolated from fresh products: Effects of nutritional and environmental conditions. *Appl. Sci.* **2021**, *11*, 3581. [CrossRef]
46. Lamas, A.; Miranda, J.M.; Vázquez, B.; Cepeda, A.; Franco, C.M. Biofilm formation, phenotypic production of cellulose and gene expression in *Salmonella enterica* decrease under anaerobic conditions. *Int. J. Food Microbiol.* **2016**, *238*, 63–77. [CrossRef]
47. Latasa, C.; Roux, A.; Toledo-Arana, A.; Ghigo, J.; Gamazo, C.; Penadés, J.R.; Lasa, I. BapA, a large secreted protein required for biofilm formation and the host colonization of *Salmonella enterica* serovar Enteritidis. *Mol. Microbiol.* **2005**, *58*, 1322–1339. [CrossRef]
48. Solano, C.; Garcia, B.; Valle, J.; Berasain, C.; Ghigo, J.; Gamazo, C.; Lasa, I. Genetic analysis of *Salmonella enteritidis* biofilm formation: Critical role of cellulose. *Mol. Microbiol.* **2002**, *43*, 793–808. [CrossRef]
49. Ledeboer, N.A.; Frye, J.G.; McClelland, M.; Jones, B.D. *Salmonella enterica* serovar Typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. *Infect. Immun.* **2006**, *74*, 3156–3169. [CrossRef]
50. Steenackers, H.; Hermans, K.; Vanderleyden, J.; De Keersmaecker, S.C. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. *Food Res Int.* **2012**, *45*, 502–531. [CrossRef]
51. Mohamed, M.; Mohamed, R.; Gharieb, R.; Amin, M.; Ahmed, H. Antimicrobial resistance, virulence associated genes and biofilm formation of salmonella species isolated from different sources. *Zagazig Vet. J.* **2021**, *49*, 208–221. [CrossRef]
52. Chuanchuen, R.; Ajariyakhajorn, K.; Koowatananukul, C.; Wannaprasat, W.; Khemtong, S.; Samngamnim, S. Antimicrobial resistance and virulence genes in *Salmonella enterica* isolates from dairy cows. *Foodborne Pathog. Dis.* **2010**, *7*, 63–69. [CrossRef]
53. Ahmed, H.A.; El-Hofy, F.I.; Shafik, S.M.; Abdelrahman, M.A.; Elsaid, G.A. Characterization of virulence-associated genes, antimicrobial resistance genes, and class 1 integrons in *Salmonella enterica* serovar Typhimurium isolates from chicken meat and humans in Egypt. *Foodborne Pathog. Dis.* **2016**, *13*, 281–288. [CrossRef] [PubMed]
54. Park, C.J.; Andam, C.P. Distinct but intertwined evolutionary histories of multiple *Salmonella enterica* subspecies. *MSystems* **2020**, *5*, e00515-19. [CrossRef] [PubMed]
55. Chia, T.W.; Goulter, R.M.; McMeekin, T.; Dykes, G.A.; Fegan, N. Attachment of different *Salmonella* serovars to materials commonly used in a poultry processing plant. *Food Microbiol.* **2009**, *26*, 853–859. [CrossRef]
56. Duffy, L.L.; Dykes, G.A.; Fegan, N. A review of the ecology, colonization and genetic characterization of *Salmonella enterica* serovar Sofia, a prolific but avirulent poultry serovar in Australia. *Food Res. Int.* **2012**, *45*, 770–779. [CrossRef]
57. Römling, U. Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. *Cell. Mol. Life Sci. CMLS* **2005**, *62*, 1234–1246. [CrossRef] [PubMed]
58. Römling, U.; Rohde, M.; Olsén, A.; Normark, S.; Reinköster, J. AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella* Typhimurium regulates at least two independent pathways. *Mol. Microbiol.* **2000**, *36*, 10–23. [CrossRef]

59. Jain, S.; Chen, J. Attachment and biofilm formation by various serotypes of *Salmonella* as influenced by cellulose production and thin aggregative fimbriae biosynthesis. *J. Food Prot.* **2007**, *70*, 2473–2479. [CrossRef]
60. Fàbrega, A.; Vila, J. *Salmonella enterica* serovar Typhimurium skills to succeed in the host: Virulence and regulation. *Clin. Microbiol. Rev.* **2013**, *26*, 308–341. [CrossRef]
61. Ju, X.; Li, J.; Zhu, M.; Lu, Z.; Lv, F.; Zhu, X.; Bie, X. Effect of the *luxS* gene on biofilm formation and antibiotic resistance by *Salmonella* serovar Dublin. *Food Res. Int.* **2018**, *107*, 385–393. [CrossRef]
62. Bhardwaj, D.K.; Taneja, N.K.; Shivaprasad, D.P.; Chakotiya, A.; Patel, P.; Taneja, P.; Sachdev, D.; Gupta, S.; Sanal, M.G. Phenotypic and genotypic characterization of biofilm forming, antimicrobial resistant, pathogenic *Escherichia coli* isolated from Indian dairy and meat products. *Int. J. Food Microbiol.* **2021**, *336*, 108899. [CrossRef]
63. Chin, K.C.; Taylor, T.D.; Hebrard, M.; Anbalagan, K.; Dashti, M.G.; Phua, K.K. Transcriptomic study of *Salmonella enterica* subspecies *enterica* serovar Typhi biofilm. *BMC Genom.* **2017**, *18*, 836. [CrossRef] [PubMed]
64. Kim, J.E.; Lee, Y.J. Antimicrobial resistance and virulence genes of *Salmonella* isolates from chicken industry. *Prev. Vet. Med.* **2017**, *41*, 189–192. [CrossRef]
65. Suez, J.; Porwollik, S.; Dagan, A.; Marzel, A.; Schorr, Y.I.; Desai, P.T.; Agmon, V.; McClelland, M.; Rahav, G.; Gal-Mor, O. Virulence gene profiling and pathogenicity characterization of non-typhoidal *Salmonella* accounted for invasive disease in humans. *PLoS ONE* **2013**, *8*, e58449. [CrossRef] [PubMed]
66. Flemming, H.C.; Wingender, J.; Szewzyk, U.; Steinberg, P.; Rice, S.A.; Kjelleberg, S. Biofilms: An emergent form of bacterial life. *Nat. Rev. Microbiol.* **2016**, *14*, 563–575. [CrossRef] [PubMed]
67. Ballén, V.; Cepas, V.; Ratia, C.; Gabasa, Y.; Soto, S.M. Clinical Escherichia coli: From biofilm formation to new antibiofilm strategies. *Microorganisms* **2022**, *10*, 1103. [CrossRef]
68. Eran, Z.; Akçelik, M.; Yazıcı, B.C.; Özcengiz, G.; Akçelik, N. Regulation of biofilm formation by marT in *Salmonella* Typhimurium. *Mol. Biol. Rep.* **2020**, *47*, 5041–5050. [CrossRef]
69. Niba, E.T.; Naka, Y.; Nagase, M.; Mori, H.; Kitakawa, M. A genome-wide approach to identify the genes involved in biofilm formation in *E. coli*. *DNA Res.* **2007**, *14*, 237–246. [CrossRef]
70. Zeiner, S.A.; Dwyer, B.E.; Clegg, S. FimA, FimF, and FimH are necessary for assembly of type 1 fimbriae on *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* **2012**, *80*, 3289–3296. [CrossRef]
71. Akcelik, N.; Akcelik, M. Characteristics and regulation of biofilm formation In. *Postępy Mikrobiol.-Adv. Microbiol.* **2021**, *60*, 113–119.
72. Cui, L.; Wang, X.; Huang, D.; Zhao, Y.; Feng, J.; Lu, Q.; Pu, Q.; Wang, Y.; Cheng, G.; Wu, M.; et al. CRISPR-cas3 of *Salmonella* upregulates bacterial biofilm formation and virulence to host cells by targeting quorum-sensing systems. *Pathogens* **2020**, *9*, 53. [CrossRef]
73. Sarjit, A.; Ravensdale, J.T.; Coorey, R.; Fegan, N.; Dykes, G.A. Survival of *Salmonella* under heat stress is associated with the presence/absence of CRISPR Cas genes and iron levels. *Curr. Microbiol.* **2021**, *78*, 1741–1751. [CrossRef] [PubMed]
74. Davies, M.R.; Broadbent, S.E.; Harris, S.R.; Thomson, N.R.; van der Woude, M.W. Horizontally acquired glycosyltransferase operons drive salmonellae lipopolysaccharide diversity. *PLoS Genet.* **2013**, *9*, e1003568. [CrossRef] [PubMed]
75. Whitfield, C.; Kaniuk, N.; Frirdich, E. Molecular insights into the assembly and diversity of the outer core oligosaccharide in lipopolysaccharides from *Escherichia coli* and *Salmonella*. *J. Endotoxin Res.* **2003**, *9*, 244–249. [CrossRef] [PubMed]
76. Zhang, J.F.; Shang, K.; Wei, B.; Lee, Y.J.; Park, J.Y.; Jang, H.K.; Cha, S.Y.; Kang, M. Evaluation of safety and protective efficacy of a waaJ and spiC double deletion Korean epidemic strain of *Salmonella enterica* serovar Gallinarum. *Front. Vet. Sci.* **2021**, *8*, 756123. [CrossRef] [PubMed]
77. Dehinwal, R.; Cooley, D.; Rakov, A.V.; Alugupalli, A.S.; Harmon, J.; Cunrath, O.; Vallabhajosyula, P.; Bumann, D.; Schifferli, D.M. Increased production of outer membrane vesicles by *Salmonella* interferes with complement-mediated innate immune attack. *Mbio* **2021**, *12*, e00869-21. [CrossRef] [PubMed]
78. Ma, Q.; Yang, Z.; Pu, M.; Peti, W.; Wood, T.K. Engineering a novel c-di-GMP-binding protein for biofilm dispersal. *Environ. Microbiol.* **2011**, *13*, 631–642. [CrossRef]