ORIGINAL ARTICLE

Susceptibility of *Salmonella enterica* Typhimurium dry surface biofilms to disinfection

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Abstract

In food preparation and manufacturing environments, surfaces contaminated with *Salmonella* can lead to outbreaks of Salmonellosis. We hypothesise that *Salmonella* resides on dry surfaces in a biofilm form leading to potential environmental persistence and transfer following contact. This is the first study reporting that *Salmonella* Typhimurium can form dry surface biofilm (DSB). Six disinfectants commonly used in the food industry were evaluated for their efficacy against the DSB. The two most efficacious formulations reduced bacterial viability in DSB by >99.99% when combined with mechanical removal (5 sec wiping; 300 g weight). Five out of six formulations significantly reduced bacterial transfer when combined with wiping. Complete eradication of *Salmonella* Typhimurium DSB was challenging, and mechanical removal was essential to produce a >99.99% reduction in bacterial viability within DSB. This study highlights a potential mode of survival of *Salmonella* Typhimurium on food-contact surfaces and DSB challenges for disinfection.

KEYWORDS
disinfection, dry surface biofilm, mechanical removal, *Salmonella*, transfer

1 | INTRODUCTION

Contamination of food preparation surfaces with food-borne pathogens is a global issue with severe consequences for human health from associated infections, especially in vulnerable groups including infants, elderly, and immunocompromised populations (Feasey et al., 2012). Salmonellosis can cause symptoms such as acute diarrhea and fever, leading to severe dehydration amid other complications. *Salmonella* was estimated to be responsible for over 1.6 million cases of food-borne illnesses in the United States in 2018, costing > $10 billion through associated medical costs and loss of productivity (USDA 2021). Several serovars of *Salmonella enterica* are frequently encountered in foods of animal origin, but also in fruits, vegetables, and spices (Wiedemann et al., 2014).

Salmonella has been reported to persist on food contact surfaces for long periods of time, despite repeated exposure to disinfectants (Corcoran et al., 2014; Rose et al., 2000). While Salmonellosis outbreaks have long been linked to food surfaces contaminated with *Salmonella* hydrated biofilms (Corcoran et al., 2014), the reporting of a new type of biofilm on environmental dry surfaces in healthcare settings (Hu et al., 2015; Ledwoch et al., 2018; Ledwoch, Dancer, et al., 2021; Vickery et al., 2012) provides an alternative explanation as to pathogens long-term survival on surfaces despite regular cleaning, sanitization, or disinfection. The characteristic of environmental dry surface biofilm (DSB) is that they cannot be detected by swabbing or contact plate when the surface is dried and that they are resistant to heat (Almatroudi et al., 2018) and disinfection in the absence of mechanical removal (Ledwoch, Magoga, et al., 2021).

Challenges associated with the elimination of DSB in the healthcare industry are likely to be similar to the food industry, combining a reduced susceptibility to disinfection and the potential for

transferability during cleaning, sanitization, or disinfection (Ledwoch, Dancer, et al., 2021; Ledwoch, Magoga, et al., 2021; Tahir et al., 2019).

Processing plants that produce low-moisture foods (LMF), including cereals, powdered milk products, and cocoa, among others, have been associated with outbreaks of food-borne disease (Flock et al., 2022; Sekhon et al., 2021). LMF plants require dry-cleaning disinfection protocols to maintain low water activity (A_w) and prevent biofilm formation, usually achieved via various mechanical removal strategies (International Food Standards. Codex Alimentarius 2015). However, despite efforts to maintain low A_w environments, products linked to LMF plants have been subjected to recalls due to contamination with food-borne pathogens including *Salmonella*, *Listeria monocytogenes*, *Clostridium botulinum*, and *Escherichia coli* (Ly et al., 2019; Podolak et al., 2017). Combining the findings given above with reports of bacteria persisting for periods extending to 10 years in LMF plants (Russo et al., 2013), we speculated that persistence of pathogens on food surfaces could be linked to DSB.

The objectives of this study were to determine the ability of *Salmonella* Typhimurium to form DSB and evaluate the efficacy of disinfectants and sanitisers used in retail food service establishments to reduce *Salmonella* DSB on surfaces and prevent bacterial transfer from DSB.

2 | MATERIALS AND METHODS

2.1 | Dry surface biofilm production

Salmonella enterica serovar Typhimurium ATCC SL1344 was streaked onto tryptone soya agar (TSA) plates from −80°C freezer stocks and incubated overnight at 37°C. Following an additional sub-culture step onto TSA, colonies were then selected and grown in tryptone soya broth (TSB) overnight at 37°C. Overnight cultures were pelleted by centrifugation at 3000 × g for 10 min and the supernatant was discarded and replaced with 10 mL tryptone saline chloride (TSC). A 10-fold dilution of the inoculum was performed using TSC as diluent. A further tenfold dilution step was performed in TSB supplemented with bovine serum albumin (BSA) at a final concentration of 0.3 g/L.

Sterile stainless steel coupons (10 mm, grade 2B finish) were placed into each well of a 24-well plate and 1 mL of the bacterial inoculum with BSA was added (wet phase). The plate was incubated at 21°C, 40% relative humidity (RH), for 48 h with orbital shaking at 1200 rpm followed by complete removal of the inoculum via pipetting and incubation of the plates at 37°C, 25% (RH), for 3 days (dry phase). The wet and dry phases were repeated until 3 cycles had been completed.

2.2 | Hydrated sedimentation biofilm

An overnight culture of *S*. Typhimurium was grown in TSB, centrifuged at 3000 × g, and the supernatant discarded. The cell pellet was resuspended in 10 mL TSB and further diluted 100-fold in additional TSB supplemented with BSA at a final concentration of 0.3 g/L. One

TABLE 1 Contact times and active ingredients of disinfectant formulations.

Disinfectant	Contact time (min)	Disclosed ingredients[a]
Formulation A	1	Anionic surfactants, Sodium hypochlorite
Formulation B	3	Quaternary ammonium compounds, Non-ionic surfactant, glycol ether, amino alcohol, glycosides
Formulation C	1	Lactic acid, Anionic surfactant
Formulation D	1	Quaternary ammonium compounds, Ethanol
Formulation E	3	Anionic surfactant, glycerin, phenoxyethanol
Formulation F	1	Sodium hypochlorite (100 ppm)

[a]Complete formulations constitute proprietary information.

mL was added to each well of a 24-well plate containing stainless steel coupons, as per the DSB growth protocol.

Biofilms were then grown for 96 h at 21°C with orbital shaking. At the end of the growth period, the media was carefully removed via pipetting and the coupons were washed with TSC to remove loosely attached bacteria.

2.3 | Formulation preparation and biofilm treatment

Six formulations (Table 1) were prepared according to manufacturer's instructions and underwent neutralizer validation according to BS EN13727 (2015) before use (data not shown).

A method developed by Ledwoch et al. (2019) to assess disinfectant efficacy on DSB viability was used for bacterial enumeration following DSB treatment. Formulations were decanted into trigger spray bottles and applied to each coupon using two sprays from a 20 cm distance at a 45° angle and left for the recommended contact time as per manufacturer's instructions (Table 1). Coupons were then transferred to tubes containing 10 mL of a neutralizing solution (L-histidine 1 g/L, L-α-lethicin 3 g/L, sodium chloride 8.5 g/L, tryptone 1 g/L, sodium thiosulphate 3 g/L, saponin 30 g/L, poysorbate-80 30 g/L) and glass beads (3 g). Following vortexing for 3 min, suspensions were serially diluted in TSC, and viable bacteria enumerated with the drop count method. Log_{10} reduction in viable bacteria was calculated relative to untreated control samples.

The susceptibility of hydrated and DSB to disinfection were evaluated with formulations A and B. The impact of mechanical removal post-treatment was explored with DSB only. Wiping was performed using a Wiperator device (based on ASTM2967 (2015) for 5 s using 500 g pressure and J-cloths® (Chicopee, The Netherlands) as the wipe substrate. The contact time for both formulations before wiping was 5 min. Performance of formulations A and B were compared to a water treated control. Additional experiments compared formulations

A-F against water treated DSB, using wiping for 5 s at 300 g pressure and contact times before wiping were recommended by manufacturers (Table 1). J-cloths were used as the wipe substrate for all tests. J-cloths were pre-sterilized by autoclave at 121°C for 20 min.

2.4 | Transfer post-treatment

Transfer of DSB was evaluated following wiping after the initial disinfectant contact time. Transfer was determined by 36 successive adpressions of the coupons (using 100 g pressure) across the surface of Dey-Engley (DE) neutralizing agar plates (Oxoid, UK; 120×120 mm). Plates were then incubated at 37°C for 24 h and the number of zones with positive growth (transfer) was recorded.

2.5 | Regrowth post-treatment

Following biofilm treatment with water or the formulations, the substrates used to wipe the coupons were immersed in 10 mL of DE neutralizing broth (Oxoid, UK) at 37°C for 24 h. Regrowth of viable cells was determined by a color change of the broth from purple to yellow.

2.6 | Scanning electron microscopy

Following treatment with the appropriate disinfectant formulation, DSB were immersed in 2.5% glutaraldehyde for 24 h and dehydrated through an ethanol series from 10 to 100%, with 10 min each step. Samples were sputter-coated with 20 nm AuPd and SEM images were acquired using a beam energy of 5 kV using an in-lens detector on a Sigma HD field gun Scanning Electron Microscope (Carl Zeiss Ltd, UK) at 10,000–50,000x magnification. Three representative fields of view were captured for each treatment.

2.7 | Statistical analysis

Three biological replicates were evaluated for each test. Two-way ANOVA with Dunnett's multiple comparisons was performed for log_{10} reduction tests, transfer tests, and data relating to regrowth of *S*. Typhimurium on coupons. One-way ANOVA Dunnett's multiple comparisons was performed for data obtained for regrowth of bacteria on wipe substrates. All treatments were compared to a water treated control. All statistical analyses were performed using GraphPad Prism®, version 9.4.0 (GraphPad Software Inc.).

# 3 | RESULTS AND DISCUSSION

3.1 | DSB enumeration

No test standard currently exists for measuring the efficacy of a disinfectant to reduce DSB on surfaces. Based upon the resistant phenotype of biofilms, particularly DSB, the pass/fail criteria applied in this study was based on the BS EN 13697 (2015) which has a 4 log_{10} pass threshold for bacteria dried on surfaces for disinfectants without mechanical action. Initial investigations examined the efficacy of formulations A and B using wiping at a pressure of 500 g. Using such a condition, both formulations with wiping action significantly reduced viable bacteria remaining on the coupons by >4 log_{10} compared to water treatment ($p < 0.0001$; Figure 1a). Without a mechanical removal step, both formulations failed to significantly reduce viable bacteria compared to the water control ($p > 0.05$). Conversely, treatment of the hydrated biofilms with formulation A without mechanical removal led to a significant reduction in viable bacteria ($p < 0.0001$; Figure 1a). Treatment of hydrated biofilms with formulation B without wiping did not significantly reduce viable bacteria remaining on the coupon ($p > 0.05$; Figure 1a). Since hydrated biofilms have previously been shown to be easily disturbed by mechanical removal compared to DSB (Parvin et al., 2023), adding a mechanical wiping step against the hydrated biofilms was not studied.

Additional experiments examined the efficacy of a panel of disinfectant formulations in combination with wiping, using a reduced wiping pressure of 300 g. As with our previous findings using a heavier weight, the use of a wiping step after a disinfectant treatment produced a significant reduction in the number of viable bacteria remaining on surface in comparison to the water treated control for all formulations, except formulation C (Figure 1b). However only formulations A and B passed the threshold of 4 log_{10} reduction in bacterial viability (Figure 1b). Without mechanical removal, none of the formulations resulted in >4 log_{10} reduction in *Salmonella* Typhimurium. Viable bacteria recovered from unwiped coupons pretreated with formulations A, D, E, and F were not significantly different to water treated coupons. This data corroborates results from other studies on different bacteria where mechanical removal was shown to be essential to appropriately reduce DSB on surfaces (Centeleghe et al., 2022; Ledwoch, Magoga, et al., 2021). Our findings support evidence that a "clean" food surface should be generated by the combined approach of mechanical removal and effective biocide use (Gibson et al., 1999).

In addition to mechanical removal, the type of formulation and particularly the active ingredient(s) play an important role in controlling *Salmonella* Typhimurium DSB. Formulation A which contains a mixture of sodium hypochlorite and anionic surfactant was the most efficacious, notably when compared to formulation F containing only 100 ppm sodium hypochlorite (Figure 1b). Chlorine based disinfectants have long been studied for the reduction on *Salmonella* species on food contact surfaces (Byun et al., 2020) and as such, the USA Food and Drug Administration recommends sanitization with chlorine bleach for the prevention of Salmonellosis. In hydrated biofilms, resistance to chlorine based disinfectants can largely be attributed to the extracellular polymeric substance (EPS), protecting the bacterial cells from the disinfectant until the concentration is high enough to be destructive to the EPS. DSB grown in the presence of organic load has also been shown to be more difficult to control with sodium hypochlorite than those without organic load (Ledwoch et al., 2019).

Formulation B which contained a combination of quaternary ammonium compounds (QACs) also proved to be efficacious against
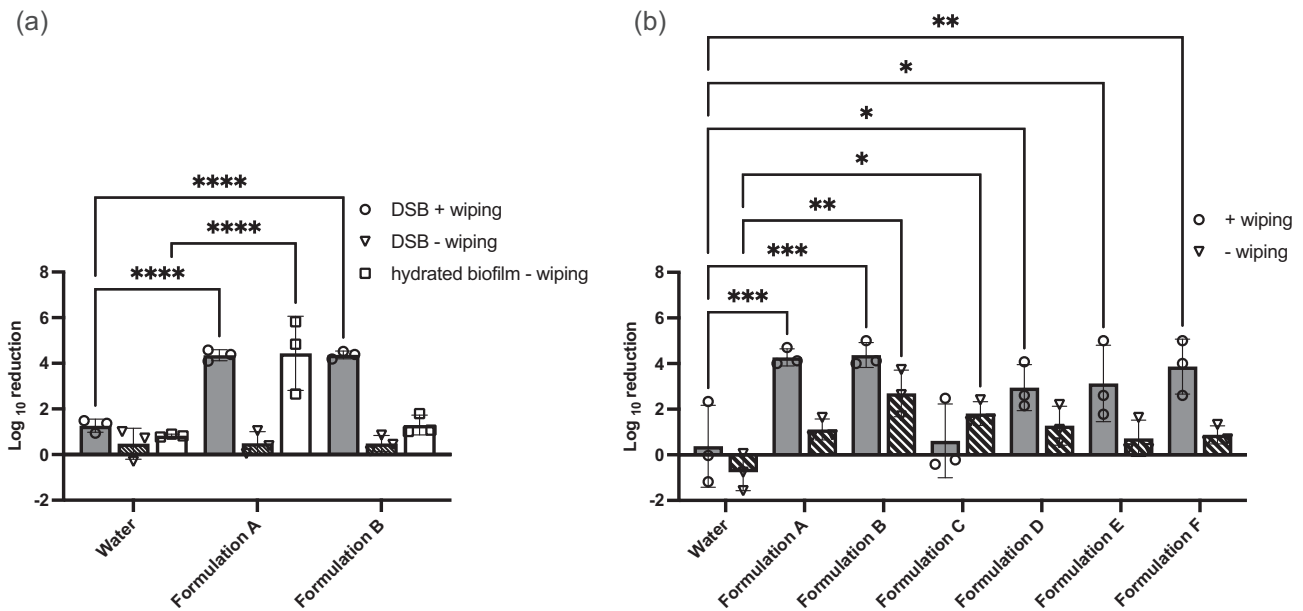
FIGURE 1 Log$_{10}$ reductions of *Salmonella* Typhimurium biofilms following treatment with disinfectants or water. Dry surface biofilm (DSB) were formed following a succession for hydrated and dried phases over a 12-day period. a. DSB and hydrated biofilms treated with water or formulations A or B for 5 min, with or without a subsequent wiping step for 5 s using 500 g pressure. Hydrated sedimentation biofilms were formed over a 96-h period. *S.* Typhimurium in hydrated biofilms were more susceptible to disinfection than DSB. b. DSB treated with water or formulations A-F for 1 min (formulations A, C, D, F, and water) or 3 min (formulation B and E), with or without subsequent wiping for 5 s using 300 g pressure. The addition of mechanical removal combined with formulations was essential to achieve a >4 log$_{10}$ reduction in viability in DSB. The type of formulation impacted efficacy overall. Means of three replicates plotted with error bars representing Standard deviation (SD). Two-way ANOVA was performed comparing treatments to a water treated control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Salmonella Typhimurium DSB, in achieving >99.99% (4 log$_{10}$) reduction of viable bacteria when mechanical action was applied. Without the addition of wiping, only a 2.7 log$_{10}$ reduction was achieved, corroborating a study by Chaves et al. (2024) where a QAC based disinfectant reduced *S. enterica* hydrated biofilm by 2 log$_{10}$. This emphasizes that QAC based formulations may be a valuable tool for reduction of *Salmonella* biofilms, including DSB, but only if combined with mechanical removal.

The negative impact of hydrated biofilms on disinfection has been well reported (Marouani-Gadri et al., 2009; Møretrø et al., 2013). Among the prominent mechanisms of resistance attributed to biofilms, the presence of extracellular polymeric substances (EPS) (Nkemngong et al., 2020), persister cells (Simões et al., 2011), and of viable but not culturable cells (VBNC) whose presence occurs in abundance in zones where nutrients are absent or limited (Ciofu et al., 2022) are worth mentioning. While the presence of EPS in DSB has been observed (Almatroudi et al., 2015; Hu et al., 2015; Ledwoch et al., 2019), the depth of the biofilm is unlikely to have a prominent role in DSB, with typical thickness reported to be only 10's of μm thick; 30 μm for *S. aureus* DSB, and 24–47 μm for environmental DSB (Almatroudi et al., 2015). Persisters have not yet been described in DSB, but viable but not culturable (VBNC) bacteria have in *Klebsiella pneumoniae* ones (Centeleghe et al., 2023).

Since our study is the first one to describe a *Salmonella* Typhimurium DSB, the reasons for the observed decreased susceptibility to disinfectants, notably in comparison to hydrated biofilms, can only be speculated currently.

Our study used contact times recommended by each product's manufacturer (Table 1), and while formulation A used a 1 min contact time, formulation B relied on a 3 min one. The impact of biocide concentration, contact time, but also soiling and type of surface, Biocide concentration, contact time, but also soiling and type of surface, impact on the efficacy of a disinfectant (Maillard et al., 2013). Here, the product concentration (apart from product F) was not disclosed, and the recommended contact times differ. Apart from products A and B, all other formulations failed to achieve a 4 log$_{10}$ reduction even with wiping despite the visible damage imparted to the bacterial cell (Figure 4).

3.2 | DSB transfer and regrowth in wipe substrate

It has been argued that the efficacy of disinfectants on surface should not only be measured as log$_{10}$ reduction but also with the absence of bacterial transfer post-treatment (Ledwoch, Magoga, et al., 2021). Two efficacy standard tests that measure the microbicidal efficacy of disinfectant products on surfaces combined log$_{10}$ reduction and

(a)

(b)
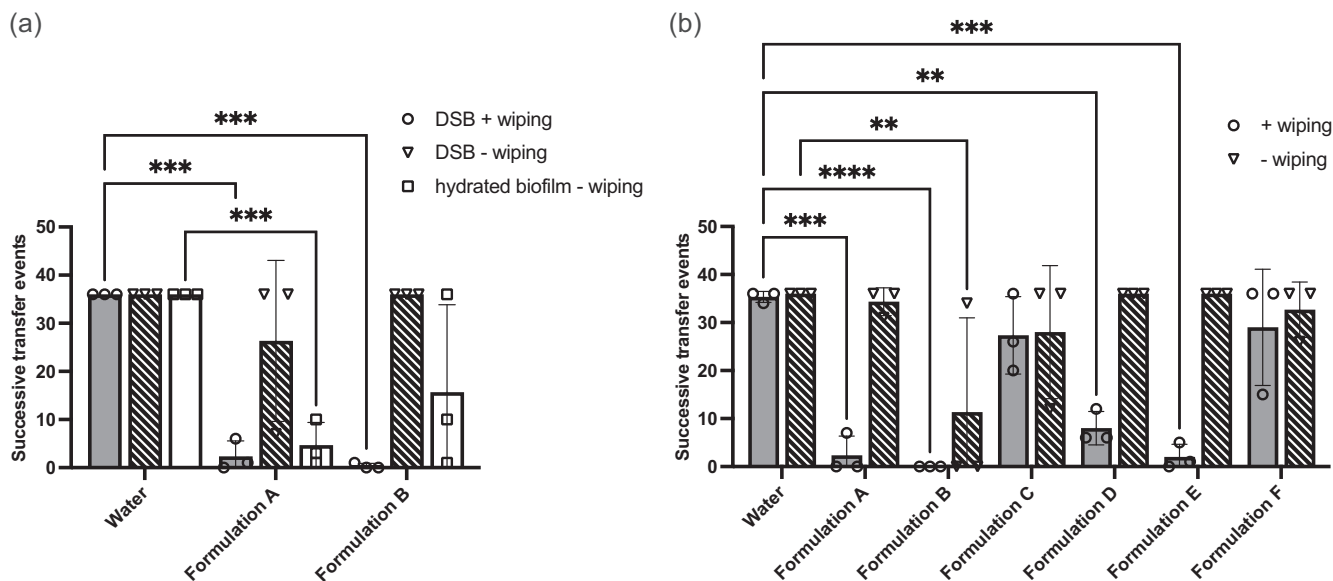
FIGURE 2 Successive transfer events of *Salmonella* Typhimurium biofilms following treatment with disinfectants or water. Dry surface biofilm (DSB) were formed following a succession for hydrated and dried phases over a 12-day period. (a) DSB and hydrated biofilms treated with water or formulations A or B for 5 min, with or without a subsequent wiping step for 5 s using 500 g pressure. There was a high number of successive transfer events from DSB compared to hydrated biofilms after treatment with formulation alone. The addition of mechanical removal was essential to reduce the number of successive transfer events. (b) DSB treated with water or formulations A–F for 1 min (formulations A, C, D, F, and water) or 3 min (formulation B and E), with or without subsequent wiping for 5 s using 300 g pressure. The type of formulation impacted the number of successive transfer events particularly in combination with wiping. Means of three replicates plotted with error bars representing SD. Two-way ANOVA was performed comparing treatments to a water treated control, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

transfer criteria (ASTM 2967-15, 2015; BS EN16615, 2015). In this study, initial investigations examined the efficacy of formulations A and B using wiping with a weight of 500 g. Using this weight, treating a surface with water did not reduce successive transfer of the *S*. Typhimurium in the biofilm regardless of the wiping action (Figure 2a). The absence of wiping did not significantly reduce bacterial transfer from DSB following exposure to either formulations A or B (Figure 2a). However, transfer of bacteria from unwiped hydrated biofilms that had been pretreated with formulation A was significantly reduced ($p < 0.001$; Figure 2a). The combination of formulation and mechanical removal significantly reduced ($p < 0.001$) the transfer of bacteria from DSB (Figure 2a).

Treatment of DSB with formulations C and F failed to significantly reduce bacterial transfer of both wiped and non-wiped surfaces. For the other formulations (A, B, D, E), mechanical removal was essential to decrease bacterial transfer (Figure 2b).

Following DSB treatment with water or formulations A or B and a subsequent wiping step, there was evidence of regrowth in all of the wipe substrates, regardless of formulation used. No statistical differences were observed between regrowth in wipes used for water treated or disinfectant treated DSB ($p > 0.05$; Figure 3). The use of disposable disinfectant wipes in the healthcare industry is widespread to aid infection prevention and control practices. Within the healthcare industry, the message "one-wipe, one-surface, one-direction, dispose (of the wipe)" has been recommended since 2009 to prevent the spread of human pathogens across surfaces (Edwards et al., 2020;
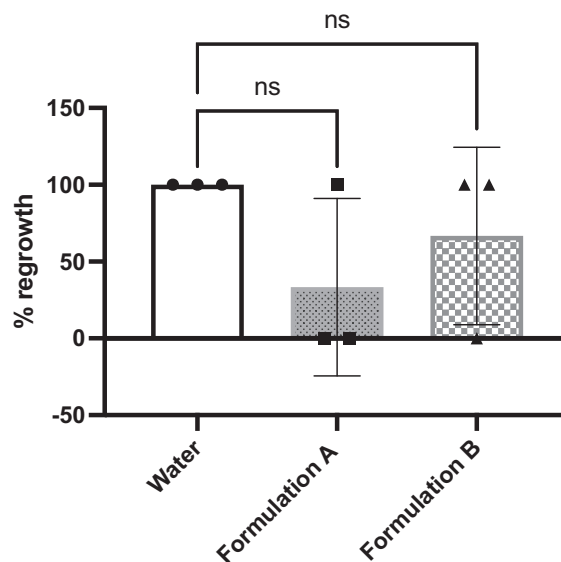
FIGURE 3 Percentage of wipe substrates with regrowth of *Salmonella* Typhimurium 24 h post-treatment of dry surface biofilm with water or formulations A or B. Wiping performed for 5 s using 500 g pressure. Overall, the formulations tested (A or B) did not affect bacterial regrowth on wipe. Means of three replicates plotted with error bars representing SD. Two-way ANOVA was performed for data set A and one-way ANOVA was performed for data set B, comparing treatments to water treated controls. ns—no statistical significance ($p > 0.05$).
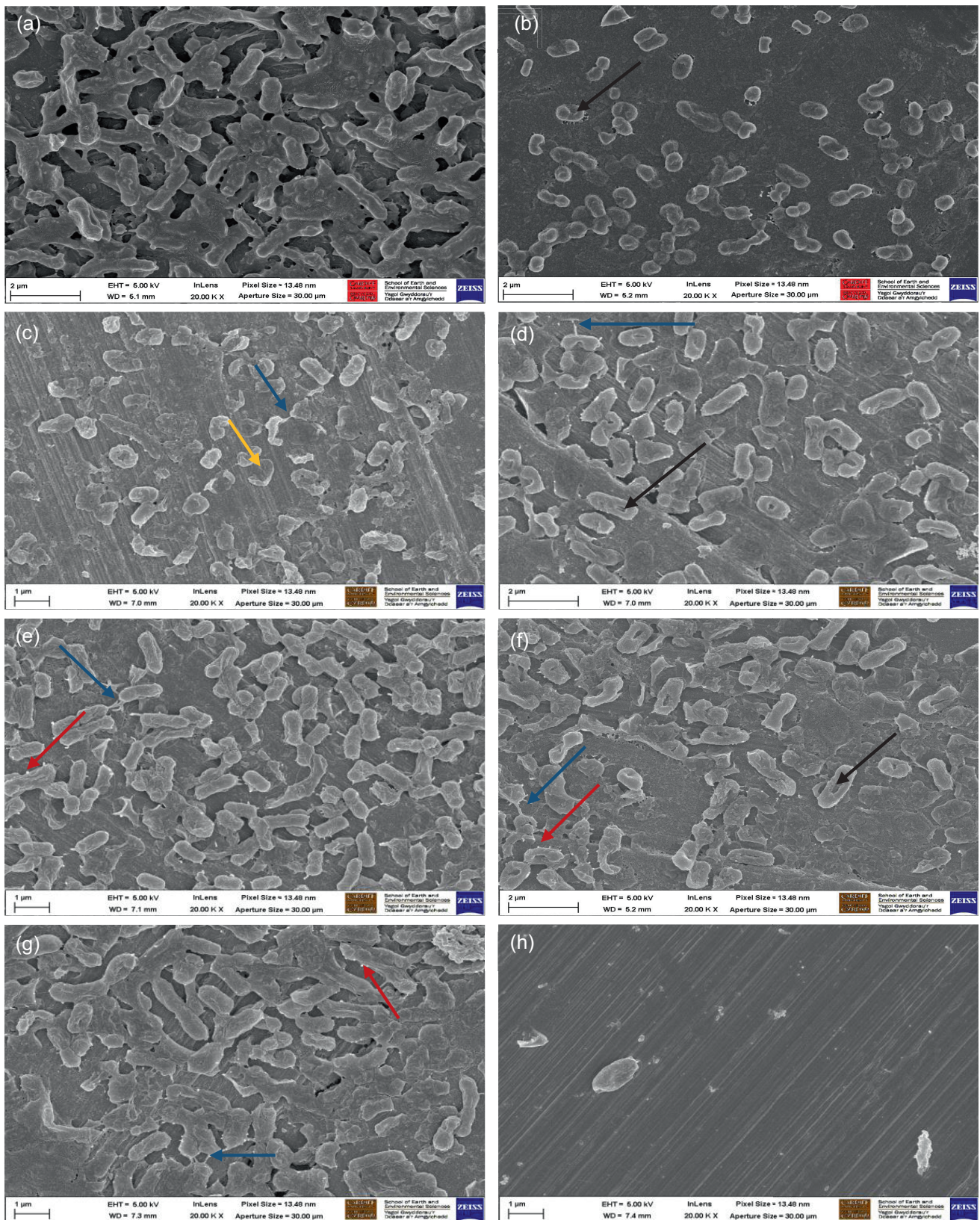
FIGURE 4 Scanning electron micrographs of *Salmonella* Typhimurium dry surface biofilm following treatment with (a). water, (b). Formulation (a, c). Formulation (b, d). Formulation (c, e). Formulation (d, f). Formulation (e, g). Formulation (f). Coupons remained unwiped. (h). Water treated coupon following wiping at 300 g pressure for 5 s. Images captured at 20,000 times magnification. Arrows indicate transmembrane tunnels, (black), membrane blebbing (red), ghost cells (yellow), and tubular appendages (blue).

Siani et al., 2011; Williams et al., 2009). With the risk of transferring bacteria from DSB following wiping, the same message would be applicable to the food hygiene industry.

3.3 | Phenotypic assessment

The study of DSB, particularly of food-borne pathogens, is a new area of investigation, and as such, the phenotypic characteristics of *S. enterica* DSB can currently only be speculated upon. Scanning electron micrographs showed notable differences in the appearance of *Salmonella* Typhimurium cells in DSB following application of disinfectant formulations compared to the water control (Figure 4). DSB treated with formulation A (Figure 4b) showed cells that appeared shortened and fuller with holes also a feature of some cells. Similar features were observed by Jawal and Lee (2014) in *S. enterica* serovar Enteritidis ghost cells and were determined to be transmembrane tunnels. Consequently, such features are also speculated to be transmembrane tunnels in this study. Formulation B treated bacteria displayed the greatest structural damage of all of the treated samples, with large amounts of cell debris present and the presence of ghost cells (Figure 4c). A notable feature of the *Salmonella* Typhimurium DSB was the presence of tubular appendages, often connecting adjacent cells. A small number of these appendages were evident in the water treated DSB, but there were notable increases in such structures following treatment with formulations B, C, and D (Figure 4c–e). Such structural damage has previously been detected in *Salmonella* hydrated biofilms (Galkina et al., 2011). Although the purpose of the increased appendages is unknown, it can be hypothesized that the treated cells increase the quantities of such structures as a protective response, with the aim of stabilizing the biofilm.

DSB treated with formulations C and E also displayed transmembrane tunnel formation, while structures speculated to be membrane blebbing are a significant feature of cells treated with formulation E (Figure 4d–f).

DSB containing coupons treated with water and wiped for 5 sec (approx. 90% reduction from surface; Figure 1a) showed that only few cells remained on the surfaces to image (Figure 4h).

4 | CONCLUSIONS

The eradication of *Salmonella* Typhimurium from surfaces of food preparation environments is essential to prevent transmission of this food-borne pathogens. Our study showed that *Salmonella* Typhimurium is capable of forming a DSB less susceptible to disinfection than hydrated biofilms. A combined action of effective disinfectant and mechanical removal is required to eliminate and prevent bacterial transfer post-treatment.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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