

Effects of environmental conditions (temperature, pH, and glucose) on biofilm formation of *Salmonella enterica* serotype Kentucky and virulence gene expression

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ABSTRACT *Salmonella* is a foodborne pathogen and an emerging zoonotic bacterial threat in the food industry. The aim of this study was to evaluate the biofilm formation by a cocktail culture of 3 wild isolates of *Salmonella enterica* serotype Kentucky on plastic (PLA), silicon rubber (SR), and chicken skin surfaces under various temperatures (4, 10, 25, 37, and 42°C) and pH values (4.0, 5.0, 6.0, 7.0, and 8.0). Then, at the optimum temperature and pH, the effects of supplementation with glucose (0, 0.025, 0.05, and 0.4% w/v) on biofilm formation were assessed on each of the surfaces. The results indicated that higher temperatures (25 to 42°C) and pH values (7.0 and 8.0) led to more robust biofilm formation than lower temperatures (4 and 10°C) and lower pH levels (4.0 to 6.0). Moreover, biofilm formation

was induced by 0.025% glucose during incubation at the optimum temperature (37°C) and pH (7.0) but inhibited by 0.4% glucose. Consistent with this finding, virulence related gene (*rpoS*, *rpoH*, *hilA*, and *avrA*) expression was increased at 0.025% glucose and significantly reduced at 0.4% glucose. This results also confirmed by field emission scanning electron microscope, confocal laser scanning microscopy, and autoinducer-2 determination. This study concluded that optimum environmental conditions (temperature 37°C, pH 7.0, and 0.25% glucose) exhibited strong biofilm formation on food and food contact surfaces as well as increased the virulence gene expression levels, indicating that these environmental conditions might be threatening conditions for food safety.

Key words: *Salmonella* Kentucky, temperature, pH, glucose, virulence gene

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INTRODUCTION

Salmonella is a genus of rod-shaped Gram-negative bacterium that belongs to the Enterobacteriaceae family. *Salmonella enterica* and *S. bongori* are the 2 types of *Salmonella* species and *S. enterica* species are classified further into 6 subspecies (Su and Chiu, 2007), with over 2,600 serotypes (Gal-Mor et al., 2014). *Salmonella* spp. are intracellular and considered as one of the major foodborne pathogens in worldwide (Jantsch et al., 2011). *Salmonella* infections are severe and can be life-threatening. Most of these infections are zoonotic, transmitted from healthy animal carriers to humans through consumption of contaminated food. Centers for Disease Control and

Prevention (CDC) reported that *Salmonella* causes approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths in the USA annually (CDC, 2020a). It also reported *Salmonella* causes foodborne diseases approximately 1 million which are associated with eggs, cucumbers, chicken, pre-cut melon, raw tuna, pistachios, sprouts, and many other foods in every year (CDC, 2020b). Poultry associated infections reported 1,722 people infected, hospitalizations 333, one death, 24% of ill younger children than 5 yr, and epidemiological evidence showed 66% people infected by contact with chicks and ducklings (CDC, 2020b).

Biofilm is a type of structural population of bacterial cells irreversibly adhered to biotic and abiotic surfaces and embedded in a self-produced matrix of extracellular polymeric substances (Nahar et al., 2018). Biofilm formation on foods and food contact surfaces is the major cause of contamination, postprocessing contamination, and cross-contamination of the final product, leading to food spoilage, product rejection, economic losses, and foodborne diseases (Giaouris et al., 2012; Srey et al.,

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2013). Biofilm formation is believed to enhance the capacity of pathogens, including pathogenic *Salmonella*, to survive and persist in the environment (Maffei et al., 2017; Sinh et al., 2018; Low et al., 2019). Besides, *Salmonella* spp. (Bezek et al., 2019), *Vibrio parahaemolyticus* (Jahid et al., 2015; Han et al., 2016; Mizan et al., 2018a), *Aeromonas hydrophila* (Jahid et al., 2015; Mizan et al., 2018b), *Enterococcus* spp. (Jahan and Holley, 2014), and *Listeria monocytogenes* (Pan et al., 2010), many bacteria can produce biofilms, but the ability varies from species to species and depends on the serotype isolate and the source of serotype (Díez-García et al., 2012; Seixas et al., 2014; Yin et al., 2018).

Biofilm formation is also influenced by environmental conditions (temperature, pH, glucose, and water activity etc.) (Moraes et al., 2018; Bezek et al., 2019). Highly diverse environmental conditions ideal for biofilm formation are encountered in the food processing environment (Wang et al., 2016; Moraes et al., 2018; Yin et al., 2018; Bezek et al., 2019). The ability of food spoilage and pathogenic bacteria to adhere to food-processing surfaces, such as stainless steel (SS), silicon rubber (SR), plastic (PLA), and food surfaces and form biofilm is a major health hazard because resistant biofilms can be a constant source of contamination (Bezek et al., 2019; Lee et al., 2020). Biofilm formation on different surfaces at different temperatures has been studied previously (Park et al., 2015; Lee et al., 2020). Biofilm formation occurs in several phases: initial substratum conditioning, reversible attachment of bacterial cells by the action of motility motion, irreversible attachment: bacterial growth and exopolysaccharides production; detachment (Srey et al., 2013).

Glucose is the most abundant simple sugar and utilizes for energy in some metabolic processes by living cells that produce biomolecules as well as encourages bacterial growth at low-nutrient environmental conditions (Jahid et al., 2013; Mizan et al., 2018a; Bezek et al., 2019). Moreover, the presence and nature of carbohydrates influence the bacterial attachment to form strong/weak biofilm in a positive or negative way (Jahid et al., 2013; Mizan et al., 2018a; Bezek et al., 2019).

Quorum sensing (QS) process is a cell to cell signaling mechanism to coordinate the expression of virulence genes and survivability for bacteria (Almasoud et al., 2016). During biofilm forming, bacteria have been shown to modulate cellular functions through QS process using signaling molecules called auto-inducers (AI) (Silagyi et al., 2009). This cell density-dependent mechanism is used by both gram-positive and gram-negative bacteria to respond to environmental stresses including nutrient deficiency, temperature effects, and host defense responses (Waters and Bassler, 2005). Moreover, AI molecules influence the expression of virulence, toxicity, sporulation, plasmid transformation, antibiotic resistance, and biofilm formation (Kendall and Sperandio, 2014). *Escherichia coli* O157:H7 and *S. typhimurium* have been shown to use the homoserine lactones (AI-1) and furanosyl borate diesters (AI-2) molecules in

a QS process (Reading et al., 2007). The bacterial biofilm formation can be impaired if autoinducer molecules activity is inhibited. QS process is used by many clinically important pathogens to monitor virulence-related processes (Galloway et al., 2011).

Responses to environmental factors impact not only growth and survival but also virulence. Bacterial stress response mechanisms are not fully understood during biofilm formation. Many studies have explored the bacterial stress response during biofilm formation by examining the gene and protein expression changes (Balamurugan, 2010; Sirsat et al., 2011; Bezek et al., 2019). The outcomes indicated that several types of shock proteins or genes are induced under stress conditions are either known or purported to protect DNA and enzymes. Some sigma factors, such as σ^S (encoded by the *rpoS* gene) and σ^H (encoded by the *rpoH* gene), are involved in stress-related gene expression (Ray and Bhunia, 2013; Bezek et al., 2019). In the stationary phase and the under changes in environmental conditions, σ^S supports the survival of *Salmonella* spp. and controls the expression of up to 50 proteins (Humphrey, 2004). Conversely, σ^H protects against cytoplasmic thermal stress by regulating the transcription of heat shock proteins (Spector and Kenyon, 2012). An unfavorable temperature might affect the *rpoS* and *rpoH* transcriptional levels, and thus the *Salmonella* spp. stress response under environmental conditions during biofilm formation (Bezek et al., 2019). In USA, more than 70% of human Salmonellosis cases have been linked with consumption of contaminated chicken, turkey, and eggs with 175, 133, and 45 illness outbreaks involving 1,003, 358, and 11 peoples, respectively (Lee et al., 2020). Salmonellosis caused by chicken consumption has been a critical issue in food safety worldwide, including Korea (Jeong et al., 2018). Poultry and poultry products are among the most common animal sources, and their consumption has risen significantly than that of other animal sources (beef and pork) over the last 50 yr (Byun et al., 2021). Simultaneously, poultry and poultry products, particularly chicken meat, are recognized as a major route of transmission of *Salmonella* spp. to humans. In case of Vietnam, India, and China, around 71.8, 9.4, and 15.8% of tested chicken meat samples were found *Salmonella* spp. positive (Li et al., 2019; Byun et al., 2021). In the large-scale chicken slaughtering and processing plant, supplied the vast majority of chicken meat consumption to consumers in surrounding areas and diarrhea infection by contaminated chicken meat consumption in human (Wang et al., 2013). *Salmonella* contamination persists throughout all stages of chicken processing, regardless of hygienic precautions taken (Salehi et al., 2016). While the intestines of poultry are thought to be the most likely source of contamination, abundant bacteria have been found on the broiler's surface and its attachment to chicken skin has been studied extensively (Salehi et al., 2016; Dewi et al., 2021). *Salmonella* may cause severe cross-contamination during the retail process, posing a serious public health risk. Furthermore, biofilm formation varies by species in

poultry houses at various temperatures (6, 20, 28, and 37°C) and under low and high nutrient conditions (Lamas et al., 2018), and it is critical to investigate the relationship between biofilm formation and environmental factors. Thus, we chose chicken skin as a food surface for this study at various temperatures and pH values because temperature is a common problem in many tropical countries.

Therefore, the aim of this study was to provide information about biofilm formation of *S. enterica* serotype Kentucky cocktail on different surfaces in the food industry by determining the effects of several environmental factors, such as temperature, pH, and glucose concentration. This study also explored the virulence factors/stress-related genes expression levels during biofilm formation, under the influence of these environmental conditions. This the novel study of *S. enterica* serotype Kentucky cocktail and there are no comparable studies on environmental conditions effects.

MATERIALS AND METHODS

Bacterial Strain, Culture, and Growth Conditions

The 3 wild strains of *S. enterica* serotype Kentucky isolated from chicken feces were used in this study. These strains were provided by Kangwon National University, Chuncheon, Korea. A 100- μ L of each stock culture (10^8 to 10^9 CFU/mL), stored at -80°C in 30% (w/v) glycerol as a cryoprotectant, was inoculated into 10-mL of tryptic soy broth (TSB; BD Difco, Sparks, NV) in a 50-mL conical tube (SPL Life Sciences Co., Ltd., Gyeonggi-do, Korea) and incubated at 37°C for 24 h in a shaking incubator (Vision Scientific, VS-8480, South Korea) at 220 rpm. An aliquot (100- μ L) of the incubated culture was pipetted into 10-mL of fresh TSB and incubated for a further 24 h. The TSB broth cultures were then centrifuged at $10,000 \times g$ for 10 min at 4°C, and the pellets were washed twice with Dulbecco's phosphate buffer saline (DPBS; Sigma-Aldrich, St Louis, MO), then ultimately resuspended in peptone water (PW; BD Diagnostics, Franklin Lakes, NJ). After that, equal volumes of each strain were mixed to construct the 3-strain cocktail of *S. enterica* serotype Kentucky. A suspension of 10^5 CFU/mL was constructed from cocktail culture and confirmed by using the enumeration method on xylose lysine deoxycholate (XLD) agar (Thermo Scientific, Oxoid, Basingstoke, UK) medium followed by incubation at 37°C for 24 h. The mixed culture cocktail of *S. enterica* serotype Kentucky was used for further experiment. The mixed culture cocktail of *S. enterica* serotype Kentucky was incubated at 5 temperatures (4, 10, 25, 37, and 42°C), pH values (4.0, 5.0, 6.0, 7.0, and 8.0) for 24 h to determine the optimum temperature and pH conditions. Afterward, various amounts of glucose (0, 0.025, 0.05, and 0.4% w/v) were added to the TSB media for making biofilm formation on different

biotic and abiotic surfaces at the optimum temperature and pH for 24 h.

Biofilm Formation on PLA, SR Surfaces, and Detachment of Biofilm Population

PLA (Egg packaging; Join Co., Ltd., Eumseong, Korea), and SR (Komax Industrial Co., Ltd., Goyang-ro, Korea) were selected as delegate surfaces used in the food industry. The PLA and SR coupons (2 cm \times 2 cm \times 0.1 cm) were processed, as described elsewhere (Lee et al., 2020). Each coupon was washed thrice with sterile distilled water (DW) and 70% ethanol to remove any residual material, oil, and bacteria. The cleaned coupons were transferred to a sealed bottle and autoclaved at 121°C for 15 min, then dried at 60°C in a dry oven without external contact exposure. For biofilm formation, 100- μ L of prepared cocktail suspension was inoculated into a sterile 50- μ L Falcon tube that contained a sterilized coupon completely submerged in 10-mL TSB. Biofilms were formed on various types of experimental coupons at various temperatures (4, 10, 25, 37, and 42°C), and pH values (4.0, 5.0, 6.0, 7.0, and 8.0) for 24 h in an incubator. After biofilm formation incubation a coupon was then rinsed with sterile DW to remove unattached or loosely attached cells, then transferred into a 50- μ L Falcon tube containing 10-mL of 0.1% PW (Oxoid) with 10 glass beads by vortex for 2 min. The removed cells were subsequently vortexed and diluted in PW for enumeration. Cell numbers were quantified by plate count method after incubation on XLD agar. The biofilm cells were denoted as Log CFU/cm².

Chicken Skin Preparation, Biofilm Formation, and Detachment of Biofilm Population

The chicken skin was purchased at a local market from Anseong, Korea. The attained chicken skin was refrigerated at 4°C until use. Chicken skin was cut into pieces 10-g (\pm 0.5 g) with sterile scissors and soaked into 70% ethanol for 10 min and washed with DW for thrice. Then chicken skin was treated with UV light in a laminar flow biosafety hood for 15 min for each side to remove from any background flora from the environment (Joo et al., 2020).

For biofilm formation, 100- μ L of prepared cocktail suspension was inoculated into sterile 50- μ L Falcon tube that contained a sterilized chicken skin piece completely submerged in 10-mL TSB adjusted to optimum pH (7.0) with various amounts of glucose (0, 0.025, 0.05, and 0.4% w/v) for biofilm formation at optimum temperature (37°C) in an incubator for 24 h. After biofilm formation incubation, each sample was rinsed with sterile DW to remove the unattached or loosely attached cells. Then samples were homogenized using a stomacher (BagMixer 400; Interscience, France) at the

maximum speed for 2 min in 24 oz (710 mL) WhirlPak filter stomacher bags containing 90 mL of 0.1 % peptone water (PW). The bacteria were counted using serial dilutions in 0.1 % PW. Using Xylose lysine deoxycholate agar (XLD agar) and incubation at 37°C for 24 h, the amount of *S. typhimurium* was determined.

Motility Assay

Swimming and swarming motility assays were performed as described previously with slight modification (Ashrafudoulla et al., 2020). To assess swimming motility, 1.5- μ L bacterial culture was spotted at the middle of a nutrient agar plate containing 0.3% of Bacto agar (BD Difco) with various amounts of glucose (0, 0.025, 0.05, and 0.4%) and then incubated at 37°C for 13 h. To measure swarming, the same amount of bacterial culture was spotted at the center of a nutrient agar plate containing 0.5% of Bacto agar (BD Difco) with various amounts of glucose (0, 0.025, 0.05, and 0.4%) and then incubated at 37°C for 24 h. The motility diameter (migration of bacteria through the agar) was evaluated in millimeters.

Autoinducer (AI)-2 Quantification by Bioluminescence

For AI-2 bioassays, *S. enterica* serotype Kentucky cocktail was grown in TSB. Following centrifugation (15,000 $\times g$ for 10 min at 4°C), the supernatants were collected and sterilized by passage through Tuffryn syringe filters (pore size 0.2 μ m). The pH was adjusted to 7.0 using a 5-N NaOH solution, and the samples were stored at -20°C. AI-2 activity was measured by a modified bioluminescence assay (Mizan et al., 2018b). *Vibrio harveyi* BB170, a mutant sensor strain that only responds to AI-2 autoinducer by producing light, was used as a reporter strain (Taga and Xavier, 2011). *Vibrio harveyi* BB120, which produces AI-1 and AI-2, was used as a positive control. Both strains were grown overnight in Luria-Bertani (LB) broth (2% NaCl) at 30°C with aeration (200 rpm). *Vibrio harveyi* BB120 was centrifuged at 15,000 $\times g$ for 10 min, and the cell-free culture supernatant was then passed through a Tuffryn syringe filter (0.2 μ m) and stored at -20°C. After overnight growth, *V. harveyi* BB170 was diluted 5,000-fold in fresh AI bioassay medium (AB) to obtain 10⁵ CFU/mL. The cell-free culture supernatant (500- μ L) of *V. harveyi* BB120 or *S. enterica* serotype Kentucky cocktail was added to the diluted *V. harveyi* BB170 (4.5-mL) in a 50-mL tube and incubated at 30°C with aeration (200 rpm) for 15 h. Next, 100- μ L aliquots were transferred to a white 96-well microtiter plate (Thermo Fisher Scientific, Kamstrupvej, Denmark). Luminescence was measured every 30 min using a luminometer (GloMax 96 Microplate Luminometer for Luminescence, Promega,

Madison, WI) and the readings were expressed as relative light units (RLU).

Visualization of Biofilms by Field Emission Scanning Electron Microscope (FE-SEM)

Salmonella enterica serotype Kentucky cocktail biofilms on food contact surfaces supplemented with glucose (0, 0.025, 0.05, and 0.4% w/v) under optimum temperature and pH were visualized using a FE-SEM (Carl Zeiss, Oberkochen, Germany). Samples were prepared by fixation and dried with dehydration. Samples were left in a 2% glutaraldehyde solution (Sigma, St. Louis, MO) for pre-fixation at room temperature for 4 h. Samples were washed thrice with PBS for 10 min for pre-fixation. Then with 2% osmium tetroxide solution (Sigma) for post-fixation was performed at room temperature for 2 h and washed thrice with PBS for 10 min. The samples were gradually dehydrated with ethanol solution (50, 60, 70, 80, and 90%) in each proportion for 10 min. Afterward, samples were treatment thrice with 100% ethanol. The samples were immersed in a mixture of ethanol and hexamethyldisilazane (Sigma) at ratios of 3:1, 1:1, and 1:3 for 15 min. A final treatment samples with 100% hexamethyldisilazane was performed thrice. The treated samples were freeze-dried to evaporate moisture and kept in a desiccator until observation. Samples were fixed on an aluminum stub with carbon tape and coated with gold-palladium. The FE-SEM instrument was operated at an acceleration voltage of 5 kV at a 5-mm working distance (Ashrafudoulla et al., 2020).

Confocal Laser Scanning Microscope (CLSM)

Salmonella enterica serotype Kentucky cocktail biofilms were grown at 37°C and pH 7.0 for 24 h in a confocal dish (SPL Life Science) containing various amounts of glucose (0, 0.025, 0.05, and 0.4% w/v). After biofilm formation, biofilm cells were washed 2 times with DW and then stained for 15 min using the Film Tracer Live/Dead biofilm viability kit containing SYTO-9 and propidium iodide (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. The confocal dish was observed under a CLSM (LSM800 Airy, Carl Zeiss, Oberkochen, Germany) using a 40X water immersion objective lens with a 488 nm argon laser set at 0.2% intensity. Dual emissions were recorded over the range of 410 to 605 nm to collect SYTO-9 (green) emission fluorescence and at 645 to 700 nm to collect propidium iodide (red) emission fluorescence (Ashrafudoulla et al., 2020).

Relative Expression of Virulence Genes by Quantitative Real-Time PCR (qPCR)

Salmonella enterica serotype Kentucky cocktail cells were used for RNA extraction. Total RNA was

Table 1. Virulence-associated primers used for Real-Time PCR.

Target genes	Primer sequences (5'-3')	Product size (bp)
<i>16S rRNA</i>	F: CAGAAGAAGCACC GGCTAAC R: GACTCAAGCCTGCCAGTTTC	167
<i>rpoS</i>	F: GAATCTGACGAAACACGCTCA R: CCACGCAAGATGACGATATG	171
<i>rpoH</i>	F: GTTTCCTTCGCCGTACTACTG R: CCACCATTTC AACCTCATCC	169
<i>hilA</i>	F: ATTAAGGCGACAGAGCTGGA R: GCAGAAATGGGCGAAAGTAA	134
<i>avrA</i>	F: GAGCTGCTTTGGTCTCAAC R: AATGGAAGGC GTTGAATCTG	173

F; Forward, R; Reverse.

extracted from the cell pellets using a commercial RNeasy Mini kit (Qiagen Co., Ltd., Anseong, Korea) according to the manufacturer's instructions. The cDNA was synthesized from the extracted RNA by reverse transcription PCR using a Maxime RT PreMix (Random Primer) kit, (iNtRON Biotechnology Co., Ltd., Gyeonggi-do, Korea). Gene expression was analyzed using a CFX real-time PCR system (Bio-Rad, Hercules, CA). Briefly, the complementary DNA sample was mixed with respective primers and Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific, Warrington, UK) in a total volume of 20- μ L. The 16S rRNA gene was used as an internal reference gene. The primers used in this study are listed in Table 1. RT-qPCR was performed using 1- μ L of cDNA as a template and 2X Real-Time PCR Master Mix. The PCR reaction protocol started with an initial denaturation at 95°C for 3 min followed by 40 cycles of annealing and extension at 95°C for 20 s, 58°C for 20 s, and 72°C for 20 s (Roy et al., 2021). The specificity of the qRT-PCR was measured using melting curve analysis. The relative quantification of specific genes was analyzed by $2^{-\Delta\Delta C_t}$ method were used. All values were normalized to 16S rRNA.

Statistical Analysis

At least 3 independent experiments were performed. The experimental data were analyzed by ANOVA using the SAS software (version 9.2; SAS Institute, Cary, NC). Significance was determined by Duncan's multiple-range test at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Temperature and pH of the Medium on Biofilm Formation

Data on the ability of *S. enterica* serotype Kentucky cocktail to form biofilms at 4, 10, 25, 37, and 42°C are shown in Figure 1. Biofilm formation was difference ($P < 0.05$) at higher temperature ($\geq 25^\circ\text{C}$) than the lower temperatures, irrespective of the surface (PLA, SR, and chicken skin). Therefore, the temperature range of 25 to 42°C can be suggested as the optimum conditions for biofilm formation by *S. enterica* serotype Kentucky

cocktail (Figure 1). In this study, didn't form biofilm at lower temperatures (4 and 10°C) with lower pH value 4.0 and biofilm formation was lower at temperatures 4 and 10°C with other pH values. During this study biofilm formation was higher at temperatures (25 to 42°C) and pH values (6.0 to 8.0) and among the all temperatures and pH values have shown optimum temperature 37°C and pH value 7.0 (Figure 1). In previous studies shown that there were no inhibition effects of temperatures and pH values on different *S. serotypes* (Lianou and Koutsoumanis, 2012; Yin et al., 2018). Different serotypes *S. Kottbus*, *S. meleagridis*, *S. derby*, *S. agona*, *S. Kinston*, *S. calabar*, *S. senftenberg*, and *S. typhimurium* biofilm formation was increased at higher temperatures (25 to 42°C) and pH values (6.0 to 8.0) but optimum temperature and pH shown 37°C and 7.0 (Yin et al., 2018). Among the different serotypes of *Salmonella* there were no differences on the biofilm formation except *S. Typhimurium*. During lower temperature incubation and the lower pH values decreased the biofilm formation on beef processing contract surfaces of *Salmonella* (Lianou and Koutsoumanis, 2012; Yin et al., 2018). In previous study also described pH has effects on *S. enterica* serotype Kentucky cocktail which are agreed with this study (Xu et al., 2010).

Similarly, noticed that higher temperatures (25 to 37°C) induced stronger biofilm formation by *V. parahaemolyticus* than 4 and 10°C, which resulted in attachment of bacterial cells as monolayers (Han et al., 2016). Neutral pH values and 20°C were the optimal conditions for biofilm formation among 99 *Salmonella* strains (18 serovars) and the quantities of biofilm varied among the serovars. Such trends are collaborated by other researchers (Karaca et al., 2013; Pagán and García-Gonzalo, 2015; Yang et al., 2016; Yin et al., 2018) and the current results (Figure 1). Incubation temperature and pH can affect many aspects that modulate cell attachment and biofilm formation, including cell physiology, cell surface properties, microbial transcriptomic profile, the solubility of food components/nutrients, and the properties of the extracellular polysaccharides, but the response varies from species to species (Karaca et al., 2013; Pagán and García-Gonzalo, 2015; Yang et al., 2016; Yin et al., 2018).

Salmonella enterica serotype Kentucky cocktail formed biofilm at significantly higher levels on the

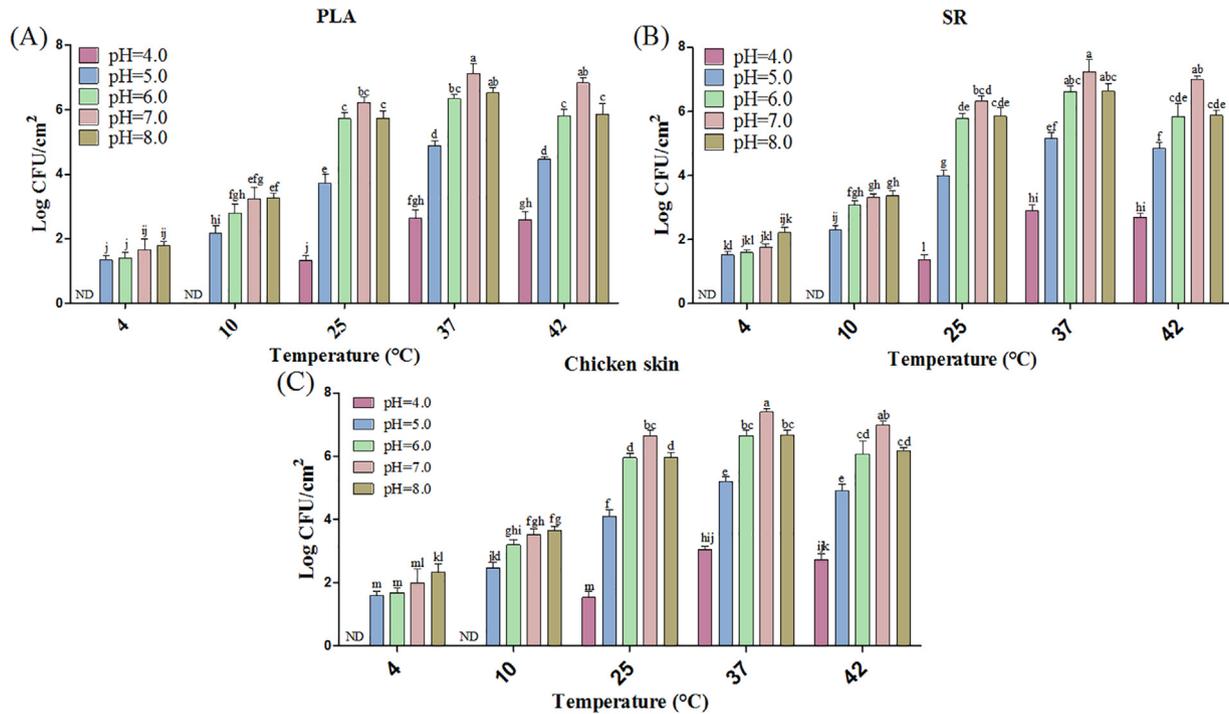


Figure 1. Biofilm formation ability of *Salmonella enterica* serotype Kentucky cocktail on plastic (PLA) (A), silicon rubber (SR) (B), and chicken skin (C) surfaces at various temperatures (4 to 42°C) and pH values (4.0 to 8.0) conditions for 24 h. Data represent mean \pm SEM of 3 independent replicates. ^{a-m}At the same temperature and pH treatment, values marked with different superscript letters are significantly different ($P < 0.05$).

chicken skin surface (almost 7.4 log CFU/cm²) (Figure 1C) than on PLA and SR surfaces (7.3 and 7.1 log CFU/cm², respectively), which produced comparable results to each other even though SR has rougher surface than PLA (Figure 1A and 1B, respectively). Surface roughness increases the area available for bacterial cell attachment. Surface irregularities promote initial colonization because such sites protect attached bacteria from external forces and encourage reversible to irreversible attachment (Lee et al., 2020). A previous study reported that a decrease in biofilm formation on SR could occur as a result of cell detachment before a new phase of colonization (Han et al., 2016). Most of the foodborne pathogens can form biofilms on various surfaces, and the characteristics of the surface influence the initial bacterial adhesion and biofilm formation and maturation (Gharechahi et al., 2012). Crab carapaces were generally more favorable than shrimp carapaces for adhesion of *Vibrio cholerae* O1 cells due to differences in the structure and composition of exoskeletons (Castro-Rosas and Escartin, 2002). Chitinolytic bacteria, including *Vibrio* spp. and *A. hydrophilia*, can degrade the chitin surface of crustaceans and utilize the chitin for extensive biofilm formation (Jahid et al., 2015). The extracellular chitinase produced by *Vibrio* spp. aids its attachment to invertebrate and zooplankton surfaces (Bignell, 1984). During the harvesting, processing, and storage of foods microbial pathogens and natural spoilage flora may grow, affecting the composition and texture of food and posing a potential health risk to susceptible consumers (Mudoh et al., 2014). *Vibrio parahaemolyticus* can grow rapidly in broth and on seafood

at temperatures ranging from 18 to 40°C (Han et al., 2016). It can also survive at refrigeration temperatures (Miles et al., 1997). It forms a strong biofilm on microtiter plates and stainless steel surfaces at 30°C (Mizan et al., 2016), whereas heat shock occurs at 42°C on stainless steel and glass surfaces (Wong et al., 2002).

The biofilm formation can be influenced by the growth environment and stress conditions (Moltz and Martin, 2005; Karaca et al., 2013; Nguyen et al., 2014). For 10 strains of *Staphylococcus aureus* incubated at various temperatures (20 to 48°C) in TSB, biofilm formation was strongest under suboptimal growth conditions (46°C), with most strains showing a growth optimum at 30°C (Rode et al., 2007). For *Escherichia coli* MG1655, biofilm formation in LB at pH 7.4 was pronounced at 25°C and can hardly be detectable at 37°C, whereas LB at pH 5.5 provided more biofilm formation at 37°C than 25°C (Mathlouthi et al., 2018).

Low pH can damage bacterial DNA and the pH gradient balance of cell membranes, which leads to the accumulation of volatile fatty acid anions in the cell (Russell and Wilson, 1996; Yin et al., 2018). When the pH value drops to between 3.0 and 5.0, polysaccharide and protein concentrations decrease quickly due to bacterial inactivation. However, polysaccharide and protein concentrations are more moderate at high pH than at low pH (Yin et al., 2018). A study conducted on *Pseudomonas aeruginosa* biofilm showed that alkaline resistance was higher than acid resistance (Zhou et al., 2014). Additionally, resistance increased in direct proportion to the increase in biofilm age (Al-

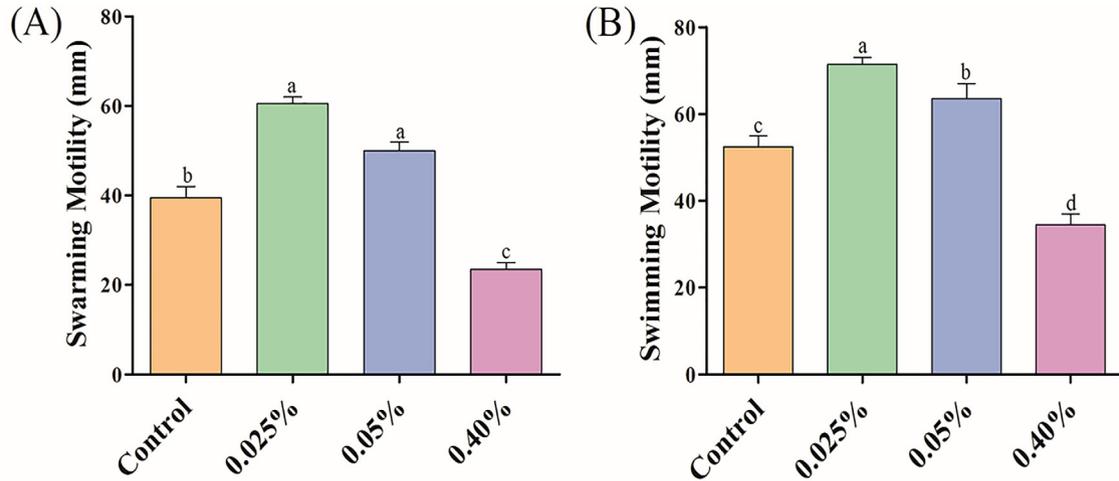


Figure 2. Swarming (A) and swimming (B) motility assay for *Salmonella enterica* serotype Kentucky cocktail at optimum conditions (37°C and pH 7.0) in the presence of various amounts of glucose (0, 0.025, 0.05, and 0.4% w/v). Data represent mean \pm the SEM of 3 independent experiments. Within each treatment, values marked with different letters (a–d) are significantly different by Duncan's multiple-range test ($P < 0.05$).

Azemi et al., 2011). The sodium proton pumps import 2 H⁺ ions for each Na⁺ ion exported. These pumps play an important role in ensuring the cell adaptation at alkaline pH (Padan et al., 2001).

Motility Assay

Motility plays an important role in biofilm formation. Glucose had a strong impact on the swimming and swarming motilities of *S. enterica* serotype Kentucky cocktail isolates at the optimum temperature (37°C) and pH (7.0) conditions (Figure 2). Glucose at 0.025% induced and at 0.4% glucose inhibited swarming and swimming motility than the control (Figure 2A, and 2B, respectively). Motility is important for biofilm formation by different pathogenic bacteria and the polar and lateral flagellar systems of pathogenic bacteria play a

crucial role in biofilm formation under different environmental stress and nutrient supplementation (Mandlik et al., 2008). It also noted that supplementation of 0.25% glucose concentration had an inhibitory effect on the motility of *A. hydrophilia*, whereas no inhibitory effect was observed with 0.05%.

Effect of Glucose on Biofilm Formation

Biofilm formation by *S. enterica* serotype Kentucky cocktail was tested at various amounts of glucose (0, 0.025, 0.05, and 0.4% w/v) during incubation at the optimum temperature and pH conditions (37°C and pH 7.0). Linear regression analysis revealed that 0.025% glucose promoted biofilm formation on PLA, SR, and chicken skin surfaces, whereas 0.4% glucose decreased number of viable cells (Figure 3). There was a stepwise increase in

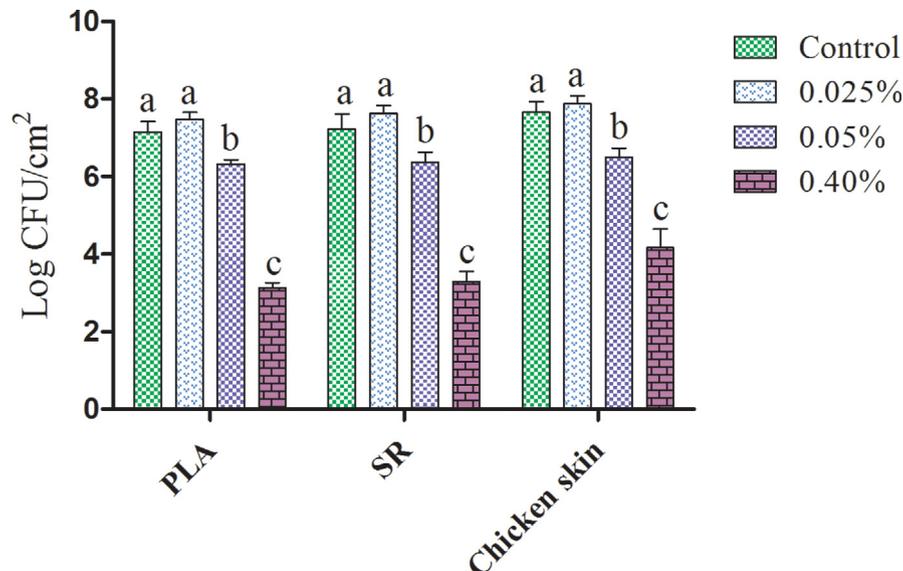


Figure 3. Biofilm formation on plastic (PLA), silicon rubber (SR), and chicken skin by *Salmonella enterica* serotype Kentucky cocktail in the presence of various amounts of glucose (0, 0.025, 0.05, and 0.4 % w/v) at optimum conditions (37°C and pH 7.0). Data represent mean \pm SEM of 3 independent replicates. Within each treatment, values marked with different letters (a–c) are significantly different by Duncan's multiple-range test ($P < 0.05$).

biofilm formation on PLA and SR surfaces up to 0.025 to 0.05% glucose, after which it decreased dramatically, regardless of the surface, with the lowest biofilm formation found at 0.4% glucose (Figure 3). Thus, 0.025% glucose was the best for biofilm formation of *S. enterica* serotype Kentucky cocktail. Glucose can enhance or inhibit bacterial growth of *S. Typhimurium* biofilm which is completely agreed with these results (Ngwai et al., 2006). The effects of glucose during biofilm formation were variable and it enhanced or inhibited the biofilm growths depending on species to species (Ngwai et al., 2006). Similarly, biofilm formation of *S. dublin* on surface enhanced and inhibited at the presence of 0.025% and 0.4% glucose, respectively (Ju et al., 2018). Some have shown that combinations of glucose and NaCl negatively influenced biofilm formation (Rode et al., 2007). Other research demonstrated that biofilm formation enhanced with glucose supplementation (Houot et al., 2010; Jahid et al., 2013), whereas notable biofilm formation was observed with limited glucose and mucin (Bowden and Li, 1997). Furthermore, 2.5% glucose inhibited initial biofilm formation but not later stages in a stage-shift biofilm assay (Houot et al., 2010; Jahid et al., 2013). In case of *Staphylococcus aureus*, show a narrow concentration of glucose plus NaCl affects biofilm formation in microtiter plates at 37°C (Lim et al., 2004).

The current results suggested that glucose has meaningful effects on *S. enterica* serotype Kentucky cocktail isolates and controls their ability to form biofilms. The biofilm-forming ability of the isolates may vary under different environmental conditions.

AI-2 Determination

The AI-2 determination in *S. enterica* serotype Kentucky cocktail supplemented with various amounts of glucose (0, 0.025, 0.05 and 0.4% w/v) at optimum temperature and pH is revealed in Figure 4. The

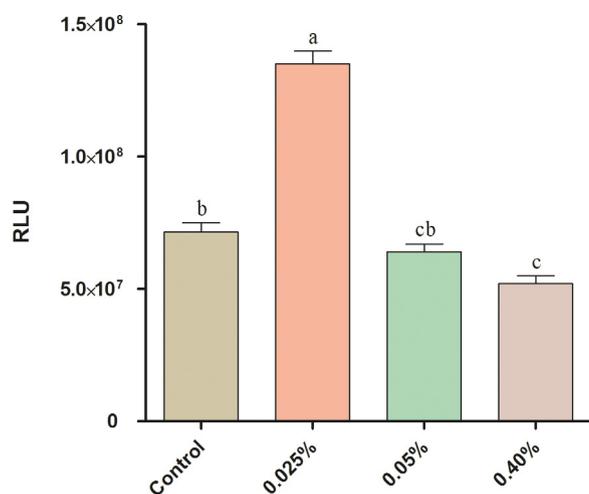


Figure 4. AI-2 production by *Salmonella enterica* serotype Kentucky isolates supplemented with various amounts of glucose (0, 0.025, 0.05, and 0.4%) at optimum conditions (37°C and pH 7.0). Data represent as mean ± SEM of 3 independent replicates. Within each treatment, values marked with different letters (a–c) are significantly different by Duncan's multiple-range test ($P < 0.05$).

production of AI-2 increased by the supplementation of 0.025% glucose and significantly ($P < 0.05$) decreased by 0.4% of glucose compared to control. *Salmonella enterica* serotype Kentucky cocktail biofilm formation is measured by quorum sensing when the bacterial population achieves a certain level with a mandatory cell density (in the presence of AI) (Henke and Bassler, 2004). During the growth of *S. enterica* serotype Kentucky especially in the stationary phase induce the target gene expression levels by reaching AI specific concentration of AI and regulates behavior of bacteria (Vendeville et al., 2005). Several bacteria were found to produce AI-2 in the presence of AI-2 in cell-free supernatants of various bacterial strains were investigated (Vendeville et al., 2005). The AI-2 was detected in *V. parahaemolyticus* by bioluminescence (Mizan et al., 2018a) and in the *V. harveyi* the growth and luminescence of are powerfully predisposed by trace elements. Glucose as well as other sugars is able to utilize by *V. harveyi* (Mizan et al., 2018a). The AI-2 bioassay completely inhibited by 0.02% glucose in response of the reporter strain to AI-2 (Mizan et al., 2018a). The AI-2 bioluminescence assay interfere with different environmental conditions such as pH, borate concentration, and glucose level (Turovskiy and Chikindas, 2006; Vilchez et al., 2007). Hence, this bioassay is a qualitative method and is not suitable for quantitative analysis (Mizan et al., 2018a).

Visual Analysis of Biofilm Formation under FE-SEM

The FE-SEM images of *S. enterica* serotype Kentucky cocktail biofilms on PLA and SR supplemented with/without glucose are shown in Figure 5, and 6. In control samples, biofilms were structurally organized with intact cell-to-cell connections. Smooth and regular cells with an intact cell membrane appeared in both the control (Figure 5A, and 6A) and with supplementation of 0.025% glucose (Figure 5B, and 6B). Conversely, the biofilm structure of 0.4% glucose supplementation samples was disrupted (Figure 5D, and 6D). The rough and irregular appearance of glucose supplementation bacterial cells showed the cells had lost their normal morphology (Figure 5C, D; and 6C, D). Glucose has shown inhibitory or enhancing effects on *S. Typhimurium* biofilm formation and which are related with this study (Ngwai et al., 2006). The effects of glucose during biofilm formation were variable and it enhanced or inhibited the biofilm growths depending on species to species (Ngwai et al., 2006). Previous study also shown biofilm formation on *S. dublin* glucose 0.02% enhanced and 0.4% glucose inhibited (Ju et al., 2018). At highest level of examined glucose amount (0.4%), glucose likely inhibited the biofilms by disrupting cell-to-cell connections and inducing cell lysis, resulting the loss of normal cell morphology (Mizan et al., 2018a). These cell-to-cell connections help in bacterial colonization and the formation of organized biofilms. Disrupting these connections induces the detachment of cells within the biofilm, which is then easily washed away (Mandlik et al., 2008).

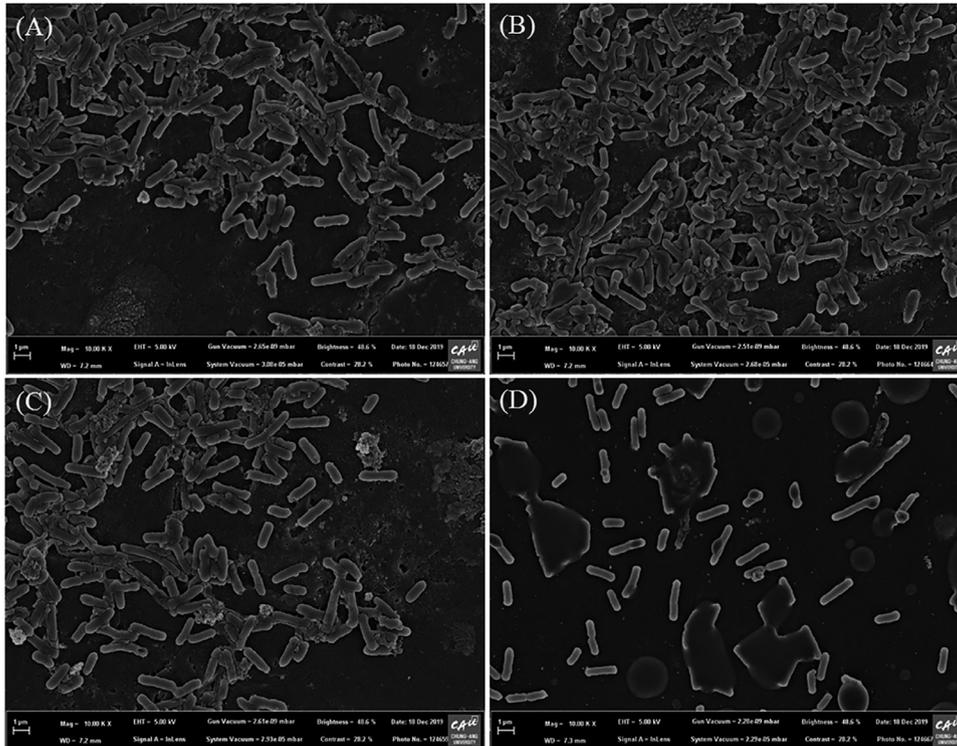


Figure 5. Representative scanning electron micrographs of *Salmonella enterica* serotype Kentucky biofilms formation in the presence of various amounts of glucose on a plastic surface at optimum conditions. (A) Control (0% glucose); (B) 0.025% glucose; (C) 0.05% glucose; (D) 0.4% glucose.

Assessing Viability of Biofilms under CLSM

CLSM has been used to analyze the structure and composition of diverse microorganisms (Bodor et al., 2011; Ma et al., 2011). The main feature of CLSM is the visualization of biological structures without affecting

the composition of microorganisms (Neu and Lawrence, 1999; Ashrafudoulla et al., 2020). In the current work, confocal imaging was used to examine effect of glucose on biofilm cells on glass surface. The control group (without glucose) biofilm (Figure 7A) appeared thick with densely populated live colonies (green color),

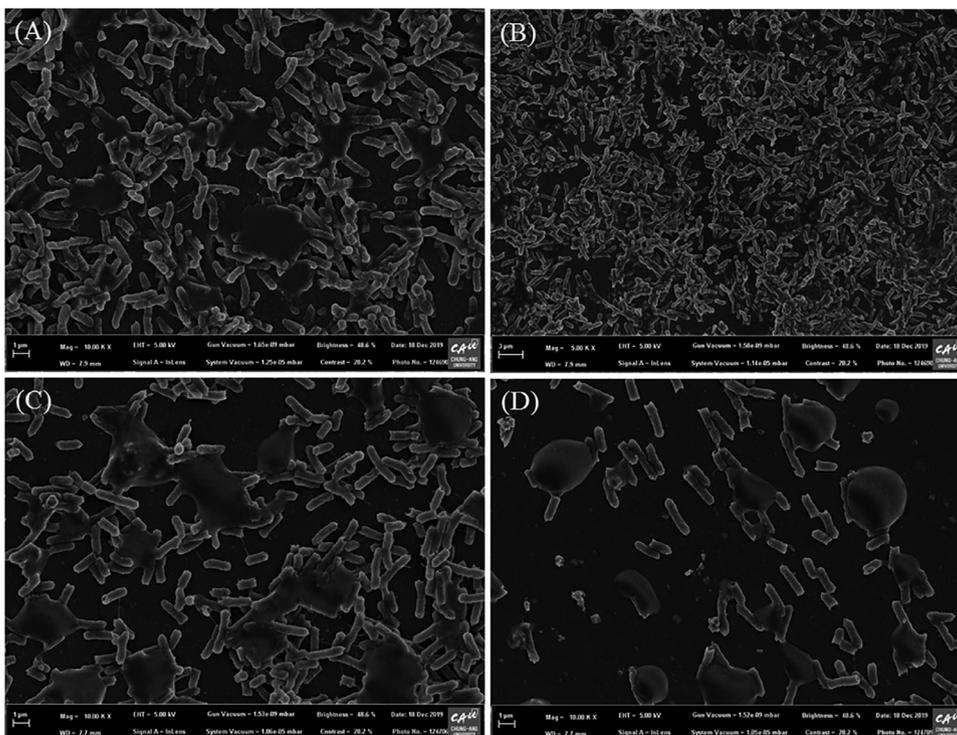


Figure 6. Representative scanning electron micrographs of *Salmonella enterica* serotype Kentucky biofilms formation in the presence of various amounts of glucose on a silicon rubber surface at optimum conditions. (A) Control (0% glucose); (B) 0.025% glucose; (C) 0.05% glucose; (D) 0.4% glucose.

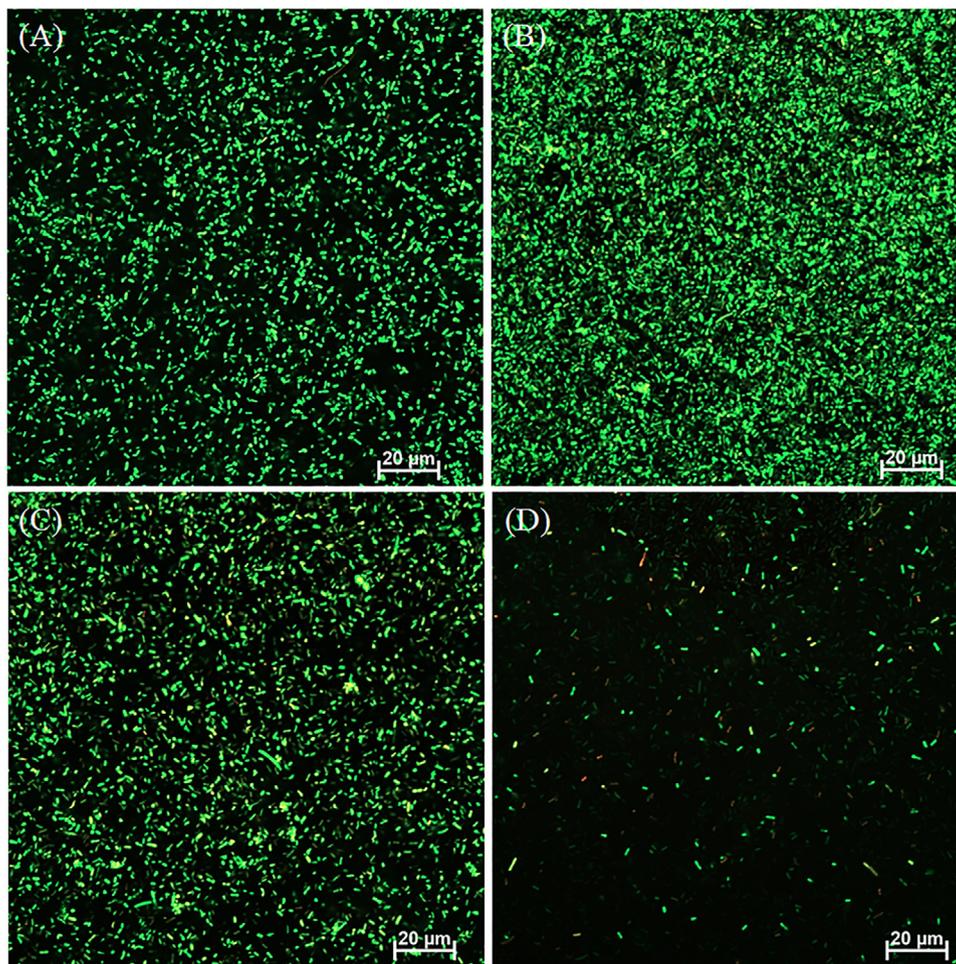


Figure 7. Representative live/dead confocal laser scanning micrographs of *Salmonella enterica* serotype Kentucky biofilms formation in the presence of various amounts of glucose at optimum conditions. Green represents live cells, and red represents dead cells. (A) Control (0% glucose); (B) 0.025% glucose; (C) 0.05% glucose; (D) 0.4% glucose.

whereas dead cells (red color) were observed in glucose supplemented samples (Figure 7B, C, and D). The visual results obtained by CLSM demonstrated that the number of green cells *S. enterica* serotype Kentucky cocktail culture increased with 0.25% and reduced with 0.4% glucose. Control samples of this study contained some dead cells (shown in red), and the number of dead cells (red) increased with increasing glucose concentration (Figure 7D). The effects of glucose during biofilm formation were variable and it enhanced or inhibited the biofilm growths depending on species to species (Ngwai et al., 2006). It also noted that biofilm formation on *S. dublin* glucose 0.02% enhanced and 0.4% glucose inhibited (Ju et al., 2018).

Relative Expression Levels of Virulence Genes by qRT-PCR

The relative expression levels of stress response genes (*rpoH* and *rpoS*), and virulence-associated genes (*avrA* and *hilA*) of *S. enterica* serotype Kentucky cocktail were analyzed by qRT-PCR and compared to the control (without glucose) samples. Relative expression levels

were significantly ($P < 0.05$) decreased with 0.4% added glucose (Figure 8). The greatest decrease in mRNA level was observed for *rpoS*, which reached an undetectable range with 0.4% added glucose followed by *avrA* and *rpoH* (Figure 8). Many genes have been identified that maintain the physiological properties, biofilm formation, quorum sensing, and overall virulence properties of *S. enterica* serotype Kentucky cocktail. Glucose can inhibit biofilm formation and provide a lethal environment to the microbes (Yang et al., 2014). The *rpoS* is an alternative sigma factor, induced in the stationary growth phase and under stress conditions (Landini et al., 2014). The stress response gene *rpoS* may play a vital role in modulating the spoilage activity of *Pseudomonas fluorescens* in food systems and observed in *E. coli*, *Salmonella*, and *Burkholderia pseudomallei* (Dong and Schellhorn, 2009; Liu et al., 2012). During the stress condition levels of reactive oxygen species can increase (Liu et al., 2012; El-Halfawy and Valvano, 2014). Similarly, during the stationary phase cells, the *rpoH* gene involved in growth and metabolism, which redirects transcription to hundreds of genes involved in multiple stress resistance, maintenance metabolism and other stationary phase functions (Weber et al., 2005) and

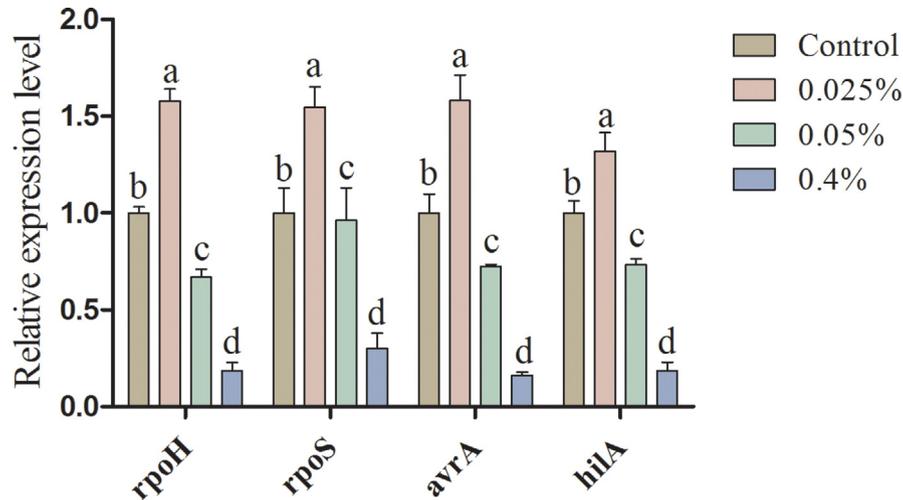


Figure 8. Relative expression levels of *rpoH*, *rpoS*, *avrA* and *hilA* genes in *Salmonella enterica* serotype Kentucky cocktail suspension supplemented with various amounts of glucose (0, 0.025, 0.05, and 0.4% w/v) at optimum conditions. ^{a-d}Different superscript letters indicate significant differences ($P < 0.05$) with 3 independent replicates.

another study showed decreased the expression of stress related genes during heat stress (Ferreira et al., 2013). High expression levels of *rpoH* and *rpoS* seem to be able to rescue the growth defects (Gu et al., 2019). The decreased transcription of the stress response genes *rpoS* and *rpoH* proved that glucose is not cell-friendly for any tested strain. It has been noted that a lethal environment can alter the pathogenicity of microbes (Yang et al., 2014). Among many virulence genes, *avrA* and *hilA* play vital roles in the defense mechanism, and host invasion by *Salmonella*, and the lysis of *Salmonella* infected microphages (Yang et al., 2014).

CONCLUSIONS

In the food industry, preventing biofilm formation is key for maintaining a high level of food safety. The results presented in the current study suggest that low temperature (4 to 10°C) and low pH values (4.0 to 6.0) can reduce the biofilm formation by *S. enterica* serotype Kentucky, whereas high temperatures (25 to 42°C) and high pH values (7.0 and 8.0) increase biofilm formation. These biofilms protect the bacterial cells against environmental conditions, making them challenging to remove. Supplementation with a low level of glucose (0.025%) increased the amount of biofilm formation, whereas 0.4% added glucose reduced biofilm formation significantly. FE-SEM and AI-2 production was observed at optimum temperature and pH combined with different amounts of glucose induced and inhibited biofilm formation. Confocal microscopy results revealed the presence of live microorganisms in biofilm even under these harsh conditions. These findings specify that glucose adjusts during biofilm formation and virulence expression levels. These results also revealed that using only conventional culture methods can give misleading results due to environmental conditions. Glucose is readily available and very cost-effective. Therefore, it could be a cost-effective approach for controlling *S. enterica* serotype Kentucky biofilm formation on biotic

and abiotic surfaces. This up-to-date study is novel and there are no comparable studies for *S. enterica* serotype Kentucky cocktail in the food industries which is very threat. Considering the problems in the health and industrial areas caused by biofilm, the development of successful control methods, while using the correct techniques to assess their efficacy, plays an important role in the fight against biofilms.

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DISCLOSURES

The authors have no competing interests to declare.

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