Introduction

Biofilms exist as summative clusters of microorganisms that could be from a single or multiple species. Biofilms are densely populated microbial communities comprising microorganisms of the same or different species that live close to each other and therefore facilitate social interaction (Davey and O’Toole 2000; Li and Tian 2012). The multicellular properties of biofilms assist in the survival of microorganisms when exposed to undesirable environmental and stressful conditions. The attachment of planktonic microorganisms to surfaces is critical for biofilm formation (Arunasri and Mohan 2019). Biofilms can be formed on food contact surfaces, contaminated food materials, natural environments such as water bodies, and on human tissues (Hall-Stoodley et al. 2004). The formation of biofilms is an important virulence factor that enhances the pathogenicity of most microbes that cause infections in humans and animals and therefore alleviate their public health significance (Costerton et al. 1999). The formation of biofilms by bacteria has resulted in increasing rates of antimicrobial resistance emerging from the potential to prevent the penetration of antibacterial agents into cells during treatment (Patel 2005) thus making biofilm control medically important. However, very few data has been reported on a substantial correlation that could exist between Salmonella serotypes isolated from chickens, the multiple antibiotic resistance behavior, incubation/storage temperature, and

**Key words:** *Salmonella*, biofilm, biofilm production potential, crystal violet microtitre
their ability to form biofilms (Diez-Garcia et al. 2012; Wang et al. 2013; Borges et al. 2018).

Similarly, the strive to achieve food safety through the inactivation of pathogenic microorganisms from food and food products is important and often faced with challenges such as biofilm formation (Sadekuzzaman et al. 2015). Microbial biofilms on food and food processing plants constitute a threat to food safety and health of consumers due to the huge tolerance to exogenous stress that results in ineffective disinfection process during plant sanitation and reduced options of antibiotics treatment, which could lead to food poisoning (Hall-Stoodley and Stoodley 2009; Sofos and Geornaras 2010). The abilities of bacteria to form biofilms have been investigated using the qualitative or the quantitative assays. In recent times, the qualitative biofilm assays have given way to the quantitative assays, which give more precise results than just findings based on observation. The quantitative biofilm assays allow for a numerical evaluation of the ability of bacteria to form biofilms. In this study, the quantitative assays were adopted based on its accuracy, reliability, and potential to enable precise quantification instruments.

Biofilm forming pathogens (Salmonella Typhimurium, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, and Staphylococcus epidermidis) have been isolated in food and food processing plants in developed and developing countries (Dourou et al. 2011; Cook et al. 2012; Wang et al. 2013; Li et al. 2017; Papa et al. 2018). Some pathogenic bacteria are capable of growing at low temperatures on food and contact surfaces. Recently, according to Webber et al. (2019) Salmonella Enteritidis have been reported to form biofilms on industrial food surfaces at relatively low temperatures (3°C). This provokes concerns for safety in cold store food preservation. Therefore it is important to research into the biofilm formation potentials of Salmonella serotypes colonizing chickens reared for food in the North West province, South Africa, which is an agricultural hub of the nation to ensure safety of foods and encourage regional trade.

Food poisoning may ensue from consuming contaminated raw, fresh, and minimally processed food commodities. Salmonella borne infection outbreaks have been associated with the ingestion of Salmonella infected livestock products such as eggs, poultry meat and pork (Hur et al. 2012; EFSA-ECDC 2018). In the European Union and the United State of America, Salmonella spp. has been implicated as the causative agent for food poisoning, which results in ill-health with many cases of the outbreak in recent years. Based on previous epidemiological studies, salmonellosis outbreaks have been traced to the food of animal origin, and research interest has been geared at investigating the occurrence of pathogenic strains of Salmonella in animal food products (Dallal et al. 2010). The rate of deaths among humans resulting from non-typhoidal salmonellosis has been increasing, especially in developing countries, and the mortality rate among children and adults in Africa ranges from 22–47% (Gordon et al. 2008). Salmonella Typhimurium is known as the main cause of foodborne salmonellosis globally, including South Africa; however, in recent years Salmonella Enteritidis have soon become the dominant cause of Salmonellosis in South Africa (Muvhali et al. 2017). From 2003 to 2007, 2013 to 2015, and October 2019 an outbreak of foodborne salmonellosis emanating from national food programme was reported in the rural areas of the KwaZulu Natal province and North West province, South Africa causing severe conditions in humans (Niehaus et al. 2011; Motladiile et al. 2019). Malangu and Ogunbanjo (2009) reported an acute Salmonella poisoning in 2005 emanating from South African Hospitals. Biofilm production was reported in drinking water (Mulamattathil et al. 2014), while Isoken (2015) reported the isolation of biofilm-forming Salmonella species in cabbage and spinach sold in South Africa. The presence of Salmonella species in food and water provides opportunities for cross-contamination along the food chain and accounts for diseases in susceptible individuals (Karkey et al. 2016; Byrd-Bredbenner, 2017). Unfortunately, investigation along the critical control points on the food value chain has not been comprehensive. Most research has focused on the retail stores, processing utensils, and processing environment (Cook et al. 2012) as a source of Salmonella contamination while few focus on the livestock rearing environment, which is critical to an effective epidemiological survey. Therefore, this research hypothesized that the incubation temperature and type of Salmonella serotypes would affect the biofilm-forming potentials of Salmonella pathogens. This will help identify the biofilm formation status of microbial communities colonizing the food environment and possibly give an explanation to the observed cases of antibiotic resistance of Salmonella serotypes so as to develop informed strategies to counteract the menace of food poisoning that could emanate from such microbial communities. The study investigated the effect of incubation temperature on biofilm-forming potentials of selected Salmonella serotypes isolated from Chickens in North-West Province, South Africa.

Experimental

Materials and Methods

Materials. The following reagents and materials were used in the study; analytical grade absolute ethanol (95%), Luria Bertani broth medium (Merck, South
Salmonella enterica

The washing process was repeated twice to enable the removal of unattached cells. A 200 µl of crystal violet dye (1% w/v) was added to each well and plates were incubated at room temperature for 1 h. After incubation, the dye was discarded, and wells were washed five times in phosphate buffer saline solution. The microtitre plate was blot dry with laboratory paper towels and was allowed to dry at room temperature. After, 200 µl of 95% ethanol was added to each well and was incubated at room temperature for 5 min. The resulting solution was thereafter transferred into a new 96 well microtitre plate. The optical density (OD) of the resulting solution was quantified in terms of absorbance at a wavelength of 630 nm in an automatic Enzyme-Linked Immunosorbent Assay (ELISA) microtitre plate reader (MB-580, Zhengzhou, China). Sterile LB broth was used as blank in the determination, while the optical densities were used to investigate the biofilm formation potential of Salmonella isolates using the following conditions as stated by Papa et al. (2018); OD<sub>C</sub> < OD<sub>C</sub> = No biofilm formation, OD<sub>C</sub> < OD<sub>C</sub> < 2OD<sub>C</sub> = Weak biofilm formation, 2OD<sub>C</sub> < OD<sub>C</sub> < 4OD<sub>C</sub> = Moderate biofilm formation, 4OD<sub>C</sub> < OD<sub>C</sub> = Strong biofilm formation; Where: OD<sub>C</sub> = OD of negative control, OD<sub>S</sub> = OD of sample. Optical densities were obtained in triplicates, and the mean obtained was regarded as optical densities for each Salmonella serotype.

Statistical analysis. The statistical analysis was done using percentages and central tendency measures such as mean and frequencies using Statistical Package for Social Sciences. The significance of the effect of incubation temperatures on biofilm formation was evaluated using the one-way analysis of variance (ANOVA). The relationship between incubation temperature and biofilm-forming potentials of Salmonella isolates was evaluated using Pearson correlation analysis. The significance of variables was evaluated at a 90% confidence interval using the Statistical Package for Social Sciences (SPSS version 17, Illinois USA).

Results and Discussion

In Table I, the identity of Salmonella serotypes used in this study is presented. The isolates were from chickens reared in North West Province, South Africa, as earlier reported by Akinola et al. (2017). The optical densities and degree of biofilm formation by Salmonella serotypes isolated from chickens as influenced by incubation temperature is as presented in Table II. The values obtained represent the optical densities obtained from the crystal violet biofilm microtitre plate assay using various Salmonella serotypes as inoculum. At incubation temperature of 25°C, the optical density of Salmonella serotypes ranged from 0.008 to 1.048 while at 37°C (0.04–1.02) and 40°C (0.023–1.509). At 37°C the OD of CHG16 (Salmonella enterica subsp. Typhimurium ATCC 14028<sup>TM</sup> and Salmonella Enteritidis ATCC 13076<sup>TM</sup> were used as positive controls, un-inoculated media broth (negative control), and an environmental strain of E. coli was used as an internal control in the experiment.
## Table I
Identities of *Salmonella* isolates used for biofilm assay.

| Isolate number | Sources           | Accession number  | Organism                                      |
|----------------|-------------------|-------------------|-----------------------------------------------|
| CHG1           | Broiler           | MG663456          | *Salmonella enterica* subsp. *enterica*       |
| CHG2           | Broiler           | MG663457          | *Salmonella enterica* subsp. *enterica*       |
| CHG3           | Broiler           | MG663458          | *Salmonella enterica* subsp. *enterica*       |
| CHG4           | Broiler           | MG663459          | *Salmonella enterica* ser. *Weltevreden*      |
| CHG5           | Broiler           | MG663460          | *Salmonella enterica* ser. *Chingola*         |
| CHG6           | Broiler           | MG663461          | *Salmonella enterica* ser. *Arizonae*         |
| CHG7           | Broiler           | MG663462          | *Salmonella enterica* ser. *Bovismorbillicans* |
| CHG8           | Layer             | MG663463          | *Salmonella enterica* subsp. *enterica*       |
| CHG9           | Layer             | MG663464          | *Salmonella enterica* subsp. *enterica*       |
| CHG10          | Layer             | MG663465          | *Salmonella enterica* ser. *Typhimurium*      |
| CHG11          | Layer             | MG663466          | *Salmonella enterica* ser. *Salamae*          |
| CHG12          | Layer             | MG663467          | *Salmonella enterica* ser. *Houten*           |
| CHG13          | Layer             | MG663468          | *Salmonella enterica* subsp. *enterica*       |
| CHG14          | Indigenous Venda  | MG663469          | *Salmonella enterica* ser. *Bareilly*         |
| CHG15          | Indigenous Venda  | MG663470          | *Salmonella enterica* subsp. *enterica*       |
| CHG16          | Indigenous Venda  | MG663471          | *Salmonella enterica* subsp. *enterica*       |
| CHG17          | Indigenous Venda  | MG663472          | *Salmonella enterica* subsp. *enterica*       |
| CHG18          | Indigenous Venda  | MG663473          | *Salmonella enterica* ser. *Heidelberg*       |
| CHG19          | Indigenous Venda  | MG663474          | *Salmonella enterica* ser. *Arizonae*         |
| CHG20          | Indigenous Venda  | MG663475          | *Salmonella enterica* subsp. *enterica*       |
| CHG21          | Indigenous Venda  | MG663476          | *Salmonella enterica* ser. *India*            |
| CHG22          | Indigenous Venda  | MG663477          | *Salmonella enterica* ser. *Crossness*        |
| CHG23          | Indigenous Venda  | MG663478          | *Salmonella enterica* ser. *Albany*           |
| CHG24          | Indigenous Venda  | MG663479          | *Salmonella enterica* ser. *Yovokome*         |
| CHG25          | Indigenous Venda  | MG663480          | *Salmonella enterica* ser. *Pullorum*         |
| CHG26          | Indigenous Venda  | MG663481          | *Salmonella enterica* ser. *Infantis*         |
| CHG27          | Broiler           | MG663482          | *Salmonella enterica* ser. *Arizonae*         |
| CHG28          | Broiler           | MG663483          | *Salmonella enterica* ser. *Heidelberg*       |
| CHG29          | Broiler           | MG663484          | *Salmonella enterica* subsp. *enterica*       |
| CHG30          | Broiler           | MG663485          | *Salmonella enterica* subsp. *enterica*       |
| CHG31          | Broiler           | MG663486          | *Salmonella bongori*                          |
| CHG32          | Broiler           | MG663487          | *Salmonella bongori*                          |
| CHG33          | Broiler           | MG663488          | *Salmonella enterica* ser. *Arizonae*         |
| CHG34          | Layer             | MG663489          | *Salmonella enterica* subsp. *enterica*       |
| CHG35          | Layer             | MG663490          | *Salmonella enterica* subsp. *Wandsworth*     |
| CHG36          | Layer             | MG663491          | *Salmonella enterica* subsp. *enterica*       |
| CHG37          | Layer             | MG663492          | *Salmonella bongori*                          |
| CHG38          | Layer             | MG663493          | *Salmonella enterica* ser. *Kentucky*         |
| CHG39          | Layer             | MG663494          | *Salmonella bongori*                          |
| CHG40          | Layer             | MG663495          | *Salmonella enterica* ser. *Blockley*         |
| CHG41          | Layer             | MG663496          | *Salmonella enterica* ser. *Newport*          |
| CHG42          | Layer             | MG663497          | *Salmonella bongori*                          |
| CHG43          | Indigenous koekoek| MG663498          | *Salmonella bongori*                          |
| CHG44          | Indigenous koekoek| MG663499          | *Salmonella enterica* ser. *Manchester*       |
| CHG45          | Indigenous koekoek| MG663500          | *Salmonella enterica* subsp. *enterica*       |
| CHG46          | Indigenous koekoek| MG663501          | *Salmonella enterica* subsp. *enterica*       |
| CHG47          | Indigenous koekoek| MG663502          | *Salmonella enterica* ser. *Typhimurium*      |
Salmonella biofilm producers from chickens

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Table I. Continued

| Isolate number | Sources               | Accession number | Organism                                      |
|----------------|-----------------------|------------------|-----------------------------------------------|
| CHG48          | Indigenous koekoek    | MG663503         | Salmonella enterica subsp. enterica          |
| CHG49          | Indigenous koekoek    | MG663504         | Salmonella enterica ser. Typhimurium         |
| CHG50          | Indigenous koekoek    | MG663505         | Salmonella enterica ser. Typhimurium         |
| CHG51          | Indigenous koekoek    | MG663506         | Salmonella enterica ser. Typhimurium         |
| CHG52          | Indigenous koekoek    | MG663507         | Salmonella enterica ser. Koessen             |
| CHG53          | Indigenous koekoek    | MG663508         | Salmonella bongori                           |
| CHG54          | Indigenous koekoek    | MG663509         | Salmonella enterica ser. Blegdam             |
| CHG55          | Indigenous koekoek    | MG663456         | Salmonella enterica subsp. enterica          |

Table II

Optical densities and degree of biofilms formed by *Salmonella* serotypes as influenced by incubation temperatures.

| ID   | *Salmonella* isolates                  | Incubation temperature | Degree of biofilms formed |
|------|----------------------------------------|------------------------|--------------------------|
|      |                                        | 25°C  | 37°C  | 40°C  | 25°C  | 37°C  | 40°C  |
| CHG1 | Salmonella enterica subsp. enterica    | 0.107 | 0.312 | 0.132 | Moderate | Weak | Moderate | Strong | Weak |
| CHG2 | Salmonella enterica subsp. enterica    | 0.075 | 0.969 | 0.342 | Moderate | Strong | Strong | Moderate | Weak |
| CHG3 | Salmonella enterica subsp. enterica    | 0.023 | 0.946 | 0.063 | No biofilm | Weak | Weak | Strong | Weak |
| CHG4 | Salmonella enterica subsp. enterica    | 0.247 | 0.271 | 0.300 | Strong | Weak | Strong | Moderate | Weak |
| CHG5 | Salmonella enterica subsp. enterica    | 0.120 | 0.291 | 0.082 | Moderate | Weak | Weak | Strong | Weak |
| CHG6 | Salmonella enterica subsp. enterica    | 0.095 | 0.319 | 0.261 | Moderate | Weak | Strong | Moderate | Weak |
| CHG7 | Salmonella enterica subsp. enterica    | 0.037 | 1.006 | 0.167 | Weak | Strong | Moderate | Weak |
| CHG8 | Salmonella enterica subsp. enterica    | 0.067 | 1.022 | 0.085 | Moderate | Weak | Weak | Moderate | Weak |
| CHG9 | Salmonella enterica subsp. enterica    | 0.405 | 1.010 | 0.082 | Strong | Weak | Weak | Strong | Weak |
| CHG10| Salmonella enterica subsp. enterica    | 0.278 | 0.885 | 0.083 | Strong | Weak | Weak | Strong | Weak |
| CHG11| Salmonella enterica subsp. enterica    | 0.149 | 0.961 | 0.077 | Strong | Moderate | Weak | Moderate | Weak |
| CHG12| Salmonella enterica subsp. enterica    | 0.303 | 0.591 | 0.112 | Strong | Moderate | Weak | Moderate | Weak |
| CHG13| Salmonella enterica subsp. enterica    | 0.039 | 1.227 | 0.010 | Weak | No biofilm | Weak | No biofilm | No biofilm |
| CHG14| Salmonella enterica subsp. enterica    | 0.026 | 0.704 | 0.065 | No biofilm | Weak | Weak | No biofilm | No biofilm |
| CHG15| Salmonella enterica subsp. enterica    | 0.800 | 0.983 | 1.098 | Strong | Weak | Strong | Strong | Weak |
| CHG16| Salmonella enterica subsp. enterica    | 0.937 | 1.017 | 1.084 | Strong | Weak | Strong | Strong | Weak |
| CHG17| Salmonella enterica subsp. enterica    | 0.259 | 1.248 | 0.407 | Moderate | Strong | Moderate | Strong | Weak |
| CHG18| Salmonella enterica subsp. enterica    | 0.341 | 1.605 | 0.395 | Strong | Weak | Strong | Strong | Weak |
| CHG19| Salmonella enterica subsp. enterica    | 0.276 | 0.561 | 0.034 | Strong | Moderate | Weak | No biofilm | No biofilm |
| CHG20| Salmonella enterica subsp. enterica    | 0.012 | 0.422 | 0.034 | No biofilm | No biofilm | No biofilm | No biofilm | No biofilm |
| CHG21| Salmonella enterica subsp. enterica    | 0.030 | 0.700 | 0.066 | No biofilm | Weak | Weak | No biofilm | No biofilm |
| CHG22| Salmonella enterica subsp. enterica    | 0.277 | 1.075 | 0.489 | Weak | Strong | Weak | Strong | Weak |
| CHG23| Salmonella enterica subsp. enterica    | 0.077 | 0.812 | 0.129 | Moderate | Weak | Moderate | Strong | Weak |
| CHG24| Salmonella enterica subsp. enterica    | 0.769 | 1.244 | 1.020 | Strong | Weak | Strong | Strong | Weak |
| CHG25| Salmonella bongori                    | 0.182 | 0.973 | 1.509 | Strong | Strong | Strong | Strong | Weak |
| CHG26| Salmonella enterica ser. Weltevreden  | 0.138 | 0.817 | 1.509 | Strong | Strong | Strong | Strong | Weak |
| CHG27| Salmonella enterica ser. Chingola      | 0.181 | 0.308 | 0.446 | Strong | Weak | Strong | Strong | Weak |
| CHG28| Salmonella enterica ser. Arizonae      | 0.108 | 0.287 | 0.198 | Moderate | Weak | Strong | Weak | Strong |
| CHG29| Salmonella enterica ser. Arizonae      | 0.151 | 0.574 | 0.152 | Moderate | Moderate | Moderate | Moderate | Weak |
| CHG30| Salmonella enterica ser. Arizonae      | 0.026 | 0.901 | 0.472 | No biofilm | Strong | Strong | Strong | Weak |
| CHG31| Salmonella enterica ser. Bovismorbificans | 0.038 | 0.848 | 0.064 | Weak | Weak | Weak | Weak | Weak |
| CHG32| Salmonella enterica ser. Bovismorbificans | 0.586 | 0.220 | 0.163 | Strong | No biofilm | Moderate | Moderate | Weak |

Serovars

| CHG4 | Salmonella enterica ser. Weltevreden | 0.138 | 0.817 | 1.509 | Strong | Strong | Strong | Strong | Weak |
| CHG5 | Salmonella enterica ser. Chingola     | 0.181 | 0.308 | 0.446 | Strong | Weak | Strong | Strong | Weak |
| CHG6 | Salmonella enterica ser. Arizonae     | 0.108 | 0.287 | 0.198 | Moderate | Weak | Strong | Weak | Strong |
| CHG7 | Salmonella enterica ser. Bovismorbificans | 0.586 | 0.220 | 0.163 | Strong | No biofilm | Moderate | Moderate | Weak |
Salmonella enterica) was highest while CHG18 (Salmonella Heidelberg) at 25°C and CHG4 (Salmonella Weltevreden) at 40°C. As expected, the negative control (un-inoculated broth) had low OD (0.267 ± 0.002) hence did not form biofilm, while the positive controls Salmonella Typhimurium (1.397 ± 0.107) and Salmonella Enteritidis (1.725 ± 0.009), and the internal control E. coli (1.236 ± 0.030) were positive to biofilm production at 24 hours of incubation. As obtained in this study, biofilm formation was greatly influenced by the Salmonella serotype colonizing the substrates than the temperature of incubation at 24 hours of incubation. The optical density of eighty percent Salmonella serotypes increased at increasing incubation temperatures of 25°C to 37°C but decreased at a higher incubation temperature of 40°C. However, the optical densities of samples CHG4, CHG5, CHG14, CHG25, CHG26, CHG45, and CHG46 increased with increasing incubation temperatures from 25°C to 37°C but decreased at a higher incubation temperature of 40°C. Similarly, the incubation temperatures had a significant effect on the optical density obtained in the positive and internal controls, while

Table II. Continued

| ID   | Salmonella isolates                     | Incubation temperature | Degree of biofilms formed |
|------|----------------------------------------|------------------------|--------------------------|
|      |                                        | 25°C | 37°C | 40°C | 25°C | 37°C | 40°C |
| CHG10| Salmonella enterica ser. Typhimurium   | 1.028 ± 0.007          | 0.230 ± 0.059           | 0.167 ± 0.166           | Strong | No biofilm | Moderate |
| CHG42| Salmonella enterica ser. Typhimurium   | 0.069 ± 0.064          | 0.089 ± 0.038           | 0.039 ± 0.018           | Moderate | Weak | No biofilm |
| CHG47| Salmonella enterica ser. Typhimurium   | 0.024 ± 0.011          | 0.920 ± 0.315           | 0.053 ± 0.026           | No biofilm | Weak | Weak |
| CHG49| Salmonella enterica ser. Typhimurium   | 0.167 ± 0.107          | 0.468 ± 0.142           | 0.163 ± 0.071           | Strong | No biofilm | Moderate |
| CHG50| Salmonella enterica ser. Typhimurium   | 0.116 ± 0.084          | 0.310 ± 0.098           | 0.099 ± 0.007           | Moderate | No biofilm | Weak |
| CHG51| Salmonella enterica ser. Typhimurium   | 0.098 ± 0.041          | 1.132 ± 0.333           | 0.185 ± 0.051           | Moderate | Weak | Strong |
| CHG11| Salmonella enterica ser. Salamae       | 0.008 ± 0.004          | 0.284 ± 0.024           | 0.173 ± 0.019           | No biofilm | Weak | Moderate |
| CHG12| Salmonella enterica ser. Houten        | 0.327 ± 0.059          | 0.360 ± 0.053           | 0.248 ± 0.118           | Strong | Weak | Strong |
| CHG14| Salmonella enterica ser. Bareilly      | 0.182 ± 0.061          | 0.906 ± 0.163           | 1.009 ± 0.642           | Strong | Strong | Strong |
| CHG18| Salmonella enterica ser. Heidelberg    | 1.048 ± 0.915          | 0.976 ± 0.104           | 0.064 ± 0.022           | Strong | Strong | Strong |
| CHG28| Salmonella enterica ser. Heidelberg    | 0.098 ± 0.012          | 0.695 ± 0.167           | 0.038 ± 0.019           | Weak | Strong | No biofilm |
| CHG21| Salmonella enterica ser. India         | 0.390 ± 0.091          | 1.024 ± 0.077           | 0.238 ± 0.094           | Strong | Strong | Strong |
| CHG22| Salmonella enterica ser. Crossness     | 0.097 ± 0.008          | 0.640 ± 0.154           | 0.402 ± 0.366           | Moderate | Moderate | Strong |
| CHG23| Salmonella enterica ser. Albany        | 0.212 ± 0.088          | 0.700 ± 0.108           | 0.303 ± 0.108           | Strong | Moderate | Strong |
| CHG24| Salmonella enterica ser. Yovokome      | 0.107 ± 0.011          | 0.906 ± 0.277           | 0.041 ± 0.014           | Weak | Strong | No biofilm |
| CHG25| Salmonella enterica ser. Pullorum      | 0.183 ± 0.082          | 0.733 ± 0.035           | 0.729 ± 0.082           | Strong | Moderate | Strong |
| CHG26| Salmonella enterica ser. Infantis      | 0.320 ± 0.115          | 0.754 ± 0.124           | 0.743 ± 0.137           | Strong | Moderate | Strong |
| CHG35| Salmonella enterica ser. Wandsworth    | 0.056 ± 0.018          | 0.723 ± 0.240           | 0.101 ± 0.031           | Weak | Weak | Moderate |
| CHG38| Salmonella enterica ser. Kentucky      | 0.214 ± 0.088          | 1.012 ± 0.224           | 0.304 ± 0.255           | Strong | Weak | Strong |
| CHG40| Salmonella enterica ser. Blockley      | 0.057 ± 0.030          | 0.387 ± 0.077           | 0.077 ± 0.037           | Weak | No biofilm | Weak |
| CHG41| Salmonella enterica ser. Newport       | 0.245 ± 0.376          | 0.604 ± 0.310           | 0.388 ± 0.554           | Strong | Weak | Strong |
| CHG44| Salmonella enterica ser. Manchester    | 0.078 ± 0.012          | 1.107 ± 0.172           | 0.128 ± 0.020           | Moderate | Weak | Moderate |
| CHG52| Salmonella enterica ser. Koessen       | 0.206 ± 0.038          | 1.021 ± 0.169           | 0.290 ± 0.034           | Strong | Strong | Strong |
| CHG54| Salmonella enterica ser. Blegdam       | 0.155 ± 0.078          | 0.584 ± 0.194           | 0.135 ± 0.027           | Strong | Moderate | Strong |
| BLNK | Blank (LB broth)                       | 0.089 ± 0.009          | 0.278 ± 0.017           | 0.0385 ± 0.036          | –     | –     | –     |
| CNTRL1|Negative control (un-inoculated broth)  | 0.025 ± 0.038          | 0.267 ± 0.002           | 0.023 ± 0.017           | No biofilm | No biofilm | No biofilm |
| CNTRL2|Positive control (Salmonella enterica ser. Typhimurium ATCC 14028™) | 0.352 ± 0.106          | 1.397 ± 0.107           | 0.493 ± 0.167           | Strong | Moderate | Strong |
| CNTRL3|Positive control (Salmonella enterica ser. Enteritidis ATCC 13076™) | 0.410 ± 0.017          | 1.725 ± 0.009           | 0.602 ± 0.059           | Strong | Moderate | Strong |
| CNTRL4|Internal Control (E. coli 0157)         | 1.031 ± 0.072          | 1.236 ± 0.030           | 1.309 ± 0.076           | Strong | Moderate | Strong |

Values represents means of triplicate determinations.
No biofilm formation (if OD<br>2OD), weak biofilm formation (if OD<br>2OD), moderate biofilm formation (2OD<br>4OD) and strong biofilm formation (4OD<br>OD). Optical density (OD) ± standard deviation at 630 nm.
CNTRL1 – Negative control (un-inoculated nutrient broth), CNTRL2 – Positive control (Salmonella enterica ser. Typhimurium), CNTRL3 – Positive control 2 (Salmonella enterica ser. Enteritidis), CNTRL4 – Positive Internal Control (Escherichia coli), BLNK – Luria Bertani broth.

25°C to 37°C but decreased at a higher incubation temperature of 40°C. However, the optical densities of samples CHG4, CHG5, CHG14, CHG25, CHG26, CHG45, and CHG46 increased with increasing incubation temperature. The optical density of the Salmonella serotype was optimum at incubation temperatures of 37°C except in isolates CHG7, CHG10 and CHG18 that were optimum at 25°C.
Salmonella biofilm producers from chickens

there was no effect on the negative control. Hence, incubation temperature and type of Salmonella serotype influences the biofilm-forming abilities of Salmonella. Biofilm formation by Salmonella serotypes are well favored at an incubation temperature of 37°C.

The degree of biofilm formed by test Salmonella serotypes is as presented in Table II. The degree of biofilms formed by Salmonella serotypes ranged from no biofilm, weak, moderate to strong biofilm. Fig. 1 presents the percent distribution of the degree of biofilm formed by selected pathogens. Salmonella serotypes that produced no biofilms ranged from 11.86% to 13.56%. The percent Salmonella serotypes that produced weak biofilms at varying temperatures ranged from 11.86% to 45.76%, and this observation was optimum at an incubation temperature of 37°C (45.76%). The percent distribution of moderate Salmonella biofilm producers at varied incubation temperatures ranged from 18.64 to 20.34% and was highest at both 25°C and 40°C (20.34%). The percent Salmonella serotype that produced strong biofilms ranged from 23.73 to 54.24% and was highest at 25°C incubation temperatures.

This study observed that biofilm production by selected Salmonella serotypes was influenced by the incubation temperature and type of Salmonella serotypes. A strong Salmonella biofilm can be produced at 25°C (room temperature) within 24 hours of incubation. An incubation temperature of 25°C favors Salmonella biofilm formation than at much higher temperatures. The ability of Salmonella serotypes to form strong biofilms at room temperatures could pose a threat to food safety and hygiene practices especially in food processing facilities. Public health pathogens, including Salmonella, has been identified to have the ability to form biofilms on food contact surfaces (Bridier et al. 2014), which supports the findings in this study. The occurrence of this Salmonella serotype in food or food contact surfaces could incur extra cost in plant sanitation, thereby increasing the overhead cost of food production, which in turn results in high food prices. Biofilm formation has been identified as one of the mechanisms of bacterial pathogens to evade antimicrobial treatment (Floyd et al. 2017). Bacteria biofilms are able to tolerate harsh conditions and resist antibiotics treatments due to a unique biofilm matrix (Sharma et al. 2019). Microbial cells can sense the extracellular environment and cause the cellular response's triggering in favor of biofilm formation (Koo and Yamada 2016). Biofilm matrices act as both physical and chemical barriers (Khan et al. 2017) that could prevent antimicrobials from reaching their targets in microbes, thus preventing the control of pathogens and increasing resistance among microorganisms implicated in biofilm formation or infections. Besides the barrier to penetration, the depletion of nutrient sources and triggering of stress response and development of biofilm resistant phenotypes in microorganisms have been proved as mechanisms that aid antibiotic resistance of pathogens (Mah and O’Toole 2001). Similarly, Salmonella pathogens have been reported to contain the alternative sigma factor (RpoS) and flagella architectures that could enable its biofilm formation (Lee et al. 1995; Kroupitski et al. 2009) which supports the biofilm formation in this study. Hence, Salmonella biofilms could pose a serious threat to the effective treatment of salmonellosis through antimicrobial.

Fig. 2, 3 and 4 presents the behavioral patterns of Salmonella serotypes to biofilm production at 25°C, 37°C, and 40°C, respectively. At 25°C, 50% of the total Salmonella enterica subsp. enterica produced strong biofilm while at 37°C and 40°C only 38.7% had strong biofilm formation. Salmonella bongori (50%) produced strong biofilm at 25°C and 40°C (33.3%) whereas could not produce strong biofilms at 37°C. Only 33.3% of Salmonella Typhimurium produced strong biofilms at 25°C, while 16.7% at 40°C. However, none of the isolate produced strong biofilms at 40°C. Furthermore, 27.8% of Salmonella enterica subsp. enterica, Salmonella Typhimurium (50%) and Salmonella bongori (16.7%) produced moderate biofilms. Also, at 37°C, Salmonella Arizona (25%) and Salmonella bongori (16.7%) produced...
moderate biofilms. However, Salmonella serotypes Crossness and Manchester could only produce moderate biofilms at 25°C and 37°C. Salmonella Pullorum, Salmonella Albany, and Salmonella Infantis could only produce moderate biofilms at 37°C, while Salmonella Bovismobificans, Salmonella Kentucky, and Salmonella Salamae produced moderate biofilms at 40°C.

Furthermore, fifty percent of total Salmonella Heidelberg, Salmonella Arizonae, Salmonella Typhimurium, and Salmonella Arizonae (25%) were weak biofilms producers at 25°C, 37°C, and 40°C, while Salmonella Yovokome, Salmonella Wandsworth, and Salmonella Blockley were all weak biofilm producers at 25°C. Weak biofilm formation by Salmonella serotypes is indicative of decreased potentials of adherence to surfaces, auto-aggregation among cells, and increased sensitivity to biocides treatments (Rendueles et al. 2013). Eleven percent of Salmonella enterica subsp. enterica, Salmonella
Salmonella biofilm producers from chickens

Typhimurium (16.7%) and Salmonella bongori (33.3%) isolates were non-biofilm producers at 25°C and 40°C while at 37°C Salmonella Typhimurium (50%) lost their biofilm producing abilities. The potentials of bacteria to form biofilms on food contact surfaces have been related to the type of media or substrate, incubation time, and type of microorganisms (Díez-García et al. 2012). The detection of biofilm-producing Salmonella serotypes isolated from chicken in this study corroborates the previous reports of Wang et al. (2013) on the occurrence and isolation of biofilm-forming Salmonella isolated from chicken processing surfaces in China. Similarly, biofilm-forming Salmonella has been isolated from tomatoes (Iturríaga et al. 2007), cereals (Cui et al. 2015), and almond (Suehr et al. 2015). The dependence of temperature and Salmonella type on the quality of biofilm formation agrees with the report of Shi and Zhu (2009) on the dependence of Salmonella type and environmental factors on the quality, quantity, and ability of Salmonella to form biofilms.

Similar to the observation made in this study, Almaguer-Flores (2013) has reported the influence of nutrient medium and bacterial cell characteristics on biofilm formation. In this study, the quality of biofilm formed by Salmonella serotypes was a function of the Salmonella serotype involved in biofilm formation. The process of biofilm formation is such a vibrant process whereby bacterium attaches itself to another cell of similar or different strains or onto surfaces, thereby producing an exopolysaccharides matrix through which they achieve survival against antibiotics or detergents (Tanaka et al. 2017). This process is affected by factors such as availability of nutrient/growth medium, pH, temperature, hydrodynamics of cells, and the hydrophobicity of contact surfaces (Irie and Parsek 2008; Dourou et al. 2011). Biofilms are extracellular polymeric substances that facilitate the interaction between bacterial cells and surfaces, which are important for the stability and survival of bacteria colonies (Olaya et al. 2013). Several authors have reported the production of biofilms in bacteria such as P. aeruginosa, S. aureus, S. epidermidis, E. coli O157:H7, Campylobacter spp. and Salmonella Typhimurium, Salmonella Enteritidis to mention but a few (Zogaj et al. 2001; Solano et al. 2002; Olaya et al. 2013; Chen et al. 2015; Yang et al. 2016; Li et al. 2017). Some strains of Salmonella Typhimurium isolated in this study do not produce biofilms, contrary to the previous report of Solano et al. (2002) on biofilm production in Salmonella Typhimurium. This observation may be due to genetic variation within the genetic make-up of Salmonella serotypes used in the investigation.

Table III presents the Pearson correlation between biofilm-forming potentials of Salmonella serotypes as influenced by incubation temperatures. The Pearson correlation coefficients ranged from 0.17 to 0.50. The correlation coefficient (r = 0.50) was highest between the biofilm-forming potentials obtained at 25°C and 40°C, indicating a significant temperature-dependent association. A positive correlation existed between the biofilm-forming potentials of Salmonella serotypes incubated at 25°C, 37°C, and 40°C. A significant positive correlation exists between Salmonella biofilm production at 25°C and 37°C (p ≤ 0.05), while a positive and
moderate correlation exists between biofilms formed at 25°C and 40°C ($p \leq 0.01$). Similarly, a positive correlation exists between biofilm formed at 37°C and 40°C at $p \leq 0.01$ with a Pearson correlation coefficient of 0.263. The closer the correlation coefficient to unity the higher the relationship that exists between variables (Benesty et al. 2009; Mukaka 2012). However, a positive correlation, as observed in this study between Salmonella biofilm formed at different incubation temperatures, is implicative of a temperature-dependent association; hence, biofilm formation in Salmonella serotypes are temperature dependent.

Microbial biofilms are composed of exopolysaccharide matrices that aid the survival and breeding of new bacteria when exposed to harsh environments (Ikuma et al. 2013). Biofilm formation is an adaptation strategy to evade antibiotics or disinfectant treatment in biofilm, producing virulent strains (Patel 2005). Biofilm formation by microorganisms could enhance pathogenicity and provoke food safety issues. Bacterial biofilms make stronger the defense systems of bacterial pathogens to antibiotic treatments (Stewart and Costerton 2001; Patel 2005). Antibiotic resistance could threaten good health, increase economic burden and poverty on both processors and consumers of food products, especially in the developing countries. The presence of selected Salmonella serotypes in foods could cause the development of biofilms, which could resist antimicrobial treatment and, thereby, cause ill-health. The control of biofilm through the use of processing plant cleaning and sanitation operations in the poultry industries has become a difficult task due to the associative resistance of Salmonella to disinfectants and antimicrobials (Merino et al. 2019). Also, the inaccessibility of antimicrobials to equipment crevices and parts has limited plant sanitation; hence, the use of well-designed and cleaning efficient equipment is important to effectively control biofilm formation (Chmielewski and Frank 2004; Merino et al. 2019). The prevention of biofilm formation still remains the best strategy to control Salmonella biofilms (Merino et al. 2019). The combined use of antimicrobials and disinfectant having a broad spectrum has been recommended for Salmonella biofilm control in the poultry plants, which resulted in the use of triclosan, nanomaterials, halogenated furanones, antibiotics, disinfectants, and quaternary ammonium salts (Bridier et al. 2011; Steenackers et al. 2012). However, Salmonella biofilms formation on food contact surfaces and food processing equipment could increase the cost of cleaning operations in plants. The increased cost of production could lead to an increased cost of food products, which affects consumers’ purchasing power, thereby casting a burden on the low- and middle-class income earners. Thus the inactivation of biofilm producers is important to ensure food safety and public health.

### Conclusions

Salmonella serotypes isolated from chickens do have the potential to produce biofilms ranging from strong to no biofilm. Salmonella Heidelberg, Salmonella enterica subspp. enterica and Salmonella Weltevreden were the highest producers of strong biofilms at 25°C, 37°C and 45°C. A significant positive correlation exists between Salmonella biofilm formation at 25°C, 37°C, and 40°C. The biofilm production potentials of Salmonella are both serotypes and temperature dependent. Ambient temperature (25°C) favors Salmonella biofilm formation than at a much higher temperature. This poses a concern to food quality and safety in homes, small and medium scale food enterprises where there is a limit to the power supply, especially in developing countries. The findings from this study are quite important for global tracking on the state of Salmonella serotypes biofilms formation and develop effective control strategies as some similar serotypes isolated from this study have been reported in other countries. The detection of strong Salmonella biofilm formers in chickens found within the North West province, South Africa, also calls for concern as biofilms forming pathogens are capable of evading antimicrobial treatment. However, a broader screening will be important to further provide information on this subject in other provinces within South Africa. Similarly, the investigation on the relationship between pathogenicity, multiple antibiotic resistance behaviors of Salmonella serotypes, and biofilm formation might be necessary to further knowledge in this field.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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