INHIBITION OF EARLY STAGES OF *SALMONELLA* TYPHIMURIUM BIOFILMS BY EXTRACELLULAR DNA (EDNA) AND GENOMIC DNA (GDNA)

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ABSTRACT

In this study, the role of extracellular DNA in the establishment and maintenance of Salmonella biofilms was investigated. As a result of spectrophotometric measurements using PicoGreen® dye, it was determined that planktonic cells contain less eDNA compared to their biofilm counterparts. As a result of incubation at 20 °C for 24h, eDNA contents of planktonic Salmonella Typhimurium SL1344 and DMC4 cells were determined as 206 ng/ml and 211 ng/ml, respectively. However, eDNA amounts increases to 313.18 ng/ml for SL1344 and 319.37 ng/ml for DMC4 at biofilm structure of the same strains. The effects of extracellular DNA (eDNA) released from biofilm cells and genomic DNA (gDNA) isolated from Salmonella cells on the biofilm formation in the bacteria were investigated using DNase I and Benzonase enzymes that digest DNA. In the experiments where both enzymes were applied, the active fragmentation of eDNA and gDNA resulted in a statistically significant ($p < 0,01$) decrease in biofilm formation at the end of 24 hours compared to non-enzyme treated samples, but this effect disappeared after 48 hours of incubation.

Keywords: Biofilm, extracellular DNA, genomic DNA, DNase, benzonase

INTRODUCTION

Biofilms are multicellular-like microbial communities characterized by cells that are irreversibly attached to biotic or abiotic surfaces, interfaces and each other via extracellular polymeric matrix (**Donlan and Costerton, 2002**). Throughout the history of microbiology, bacteria were thought to be planktonic and non-social living things. However, contrary to this, microorganisms living in nature, mostly form a biofilm structure (**Nadell *et al.,* 2008**). Biofilm-forming microbial cells are called habitat formers (**Corning, 2002; Jones *et al.,* 1994**) because they form a complex but co-ordinated living space in a three-dimensional, fungal-like structure with their extracellular polymeric matrix (EPS). The architectural structure, cell activities and the ability of the cells to organize themselves occur simultaneously during the biofilm formation process. Biofilms can occur on the biotic and abiotic surfaces in nature, such as water systems, food production surfaces, mucosal surfaces of host organisms, rocks, glaciers, food and food packaging surfaces, implants and tooth surfaces. Because biofilms constitute a source of high persistence and recontamination, they cause serious medical and industrial problems (**Gupta *et al.*, 2016**). The formation of this biogenic habitat is a dynamic process and biofilms acquire new features with the matrix produced. By producing matrices, bacteria produce a physically completely different living space by providing a shelter for self-protection, accelerating the accumulation of nutrients, a change in the biophysical physicochemical environment, and interactions between organisms in both co-operation and competition. Thus, microorganisms in the biofilm provide homeostasis even in the most severe and unfavorable environmental conditions (**Flemming *et al.*, 2016; Singer *et al.*, 2010**).

The organization of the EPS molecules depends on the interaction between the matrix-forming components and on the physiological activities of the organisms in the biofilm. EPS molecules mediate the formation of biofilm mimics. This process, which biofilm-forming microcolonies produce by organizing EPS, is continuous and dynamic. Channels and pores, which are heavily observed in the biofilm architecture, allow water, water-soluble nutrients and ions to be transported between microcolonies. Thus, a circular system is formed within biofilms (**Patel, 2005**). While 97% of the extracellular matrix constitutes water, 3% constitutes water-insoluble cellulose, fimbriae, pili flagella and amyloids with exopolysaccharides, surfactants, proteins, and eDNA in the water-soluble gel structure (**Flemming, 2016**). These components are generally involved in the adhesion of bacterial cells and in the formation of biofilms (polysaccharides), communication between biofilm-forming bacterial cells (proteins), bacterial virulence properties (glycolipids), horizontal gene transfer between bacterial cells, regulation of surface hydrophobicity, formation of skeleton of biofilm architecture (eDNA) (**Gupta *et al.*, 2016**).

Determining that the biofilm eDNA is not only derived from cell disruption, but also because it is actively secreted from living cells in the biofilm structure, has led to the idea that this component is important for biofilm (**Das *et al.*, 2013**). In studies conducted with different bacterial strains, it has been suggested that eDNA plays an important role, especially in the initial attachment phase of biofilm formation, promotion of horizontal gene transfer, development of antibiotic resistance by activating of two-component regulatory systems and maintenance of structural integrity in the mature biofilms (**Doroshenko *et al.*, 2014; Mulcahy *et al.*, 2008; Okshevsky and Meyer, 2015**). Furthermore, it has been determined that curli fibers and eDNA are tightly bound in biofilm structures and that curli-eDNA composites have a synergetic effect in triggering antibody production, leading to adaptive and innate immune systems activation (**Gallo *et al.*, 2015**). This finding is also of great significance in terms of clarifying the role of eDNA in biotic/abiotic surface adhesions and cell to cell contact at the initial stage of biofilm formation (**Doroshenko *et al.*, 2014; Johnson *et al.*, 2013; Lewenza, 2013; Mulcahy *et al.*, 2008**).

Salmonella is a genus that contains zoonotic pathogen species commonly found in the gastrointestinal tract of domestic, wild, or manufactured warm-blooded animals. Because of this feature, many ways allow *Salmonella* to be entered into the food chain. In the report published by the European Food Safety Authority (EFSA) in 2010, it is stated that one of the most important agents of foodborne outbreaks is *Salmonella* and 99 % of these outbreaks originate from biofilm forms rather than the planktonic forms of the pathogen (**Corcoran *et al.*, 2014**). For this reason, the identification of effective factors on the ability to form biofilms in *Salmonella* serovars has become the focus of intensive research. Although much research has been done on the structural and functional activity of eDNA in the biofilms of different bacterial groups, there is only one study among the members of *Salmonella* in this regard (**Wang *et al.*, 2014**). In that study, which aimed to determine the effect of eDNA on the biofilms of *S.* Typhimurium SR-11 and *S.* Typhi ST6 strains, the developed biofilms of *Salmonella* strains in presence of both DNase I and extracellular DNA were compared to control biofilms, developed without any agents. Findings indicated that 6, 12, 24 and 48 h biofilms developed in the presence of extracellular DNA were significantly weaker than the control group. On the other hand, when three-

dimensional images of biofilms developed in liquid-air interface on glass discs in the presence of 50 μg/ml and 100 μg/ml DNase I and without DNase I were examined, DNase I biofilms were found to be thicker than those that enzymes were not applied, and biofilm masses were found to be significantly increased regardless of the enzyme concentration **(Wang et al., 2014)**.

In our study, the relationship between the ability of *S.* Typhimurium to produce biofilm on polystyrene surfaces with eDNA and genomic DNA (gDNA) was investigated and it was aimed to determine whether this relationship is specific to eDNA presence.

MATERIALS AND METHODS

Bacterial Strains

Two *Salmonella* strains (*S.* Typhimurium SL1344 and DMC4) used in the research were obtained from the culture collections of the Biology Department of the Faculty of Science, Ankara University. Stock cultures were stored in Luria-Bertani (LB) liquid medium supplemented with 60% glycerol at -80 °C. Cultures were assayed for biofilm after overnight incubation at 37 °C in LB broth medium (Merck, Germany) without NaCl overnight after being **(Römling et al., 1998)**.

Biofilm Production

Biofilm formation of *Salmonella* strains was performed in 96-well microdilution plates, according to the method described by Vestby et al. (2009). Briefly, overnight *Salmonella* cultures were diluted in LB without NaCl (LB^{-NaCl}) to a final concentration of 10^5 CFU/mL. A 30 μl of this inoculum was transferred into each well of the plate, containing 100 μl LB^{-NaCl}. The incubation of plates was performed at 20 °C, at static conditions for a desired period. Following the incubation, liquid medium and bacteria were aspirated from the wells, non-adherent bacteria were gently removed by washing with phosphate buffered saline (PBS). The biofilm structures were stained at room temperature for 30 min by 0,1% crystal violet dye after being fixed with 130 μL of 98% methanol. Bounded crystal violet was solubilized by adding 200 μl of ethanol-acetone (80:20, wt/wt) and spectrophotometric measurements were done at OD$_{595}$ nm. The effects of nucleic acids, degraded by enzyme application, on biofilm formation were determined by comparing the OD values of enzyme treated and untreated samples.

eDNA and gDNA Isolations From Planktonic and Biofilm Cells

S. Typhimurium SL1344 and DMC4 strains were grown in LB broth at 37 °C with 200 rpm agitation for eDNA isolation. Following the incubation, bacterial cells were removed by centrifugation at 5,000 × g for 10 min. Phenol: chloroform precipitation method was also used in the isolation of eDNA from supernatants of centrifuged planktonic *Salmonella* cells. Quantification of isolated eDNA was performed using PicoGreen® dye according to the manufacturer's instructions (Promega, USA).

Overnight bacterial cultures (approximately 1 x 10^8 CFU/mL) were inoculated on 35-mm Petri plates containing 4 ml LB^{-NaCl} and incubated at 20 °C. Plates were incubated for 4, 12 and 24 hours for present time-dependent eDNA production levels of test strains. At the end of the incubation periods, the supernatant was removed from the medium, and the biofilm structure formed in petri dishes was carefully washed 3 times with sterile PBS, to eliminate the unbound bacteria. The biofilm structure formed in the Petri plate walls was scraped off and resuspended in 500 μl PBS, then transferred to glass tubes with three sterile glass beads and vortexed to disintegrate the matrix structure. The biofilm matrix was removed by centrifugation at 5,000 × g for 10 min and the supernatant was filtered using 45 μm pore size membranes. eDNA was isolated by phenol: chloroform precipitation and eDNA isolation was performed as described by **Tang et al. (2013)**. Phenol: chloroform precipitation method was also used in the isolation of eDNA from supernatants of centrifuged planktonic *Salmonella* cultures in LB^{-NaCl}.

The isolation of gDNA from *Salmonella* planktonic cell cultures was performed by the CTAB extraction method **(Wilson, 2000)**. One μl of the isolated DNA sample and 1 μl of PicoGreen® dye (previously diluted in 199 μl of TE buffer) were mixed concentrations were measured by spectrophotometer (LightCycler 480/Roche). These experiments were carried out with 3 parallels and 3 repetitions **(Tang et al., 2013)**.

The Effect of eDNA and gDNA on Biofilm Production

Experiments to determine the role of eDNA and gDNA on *Salmonella* biofilm structures were carried out in 96-well microtiter plates. Isolated nucleic acids (eDNA and gDNA) were resuspended in sterile distilled water to reach 20, 50 and 100 μg/mL. Each concentration of nucleic acids was then simultaneously treated with two different nuclease enzymes, DNase I and Benzonase® (final concentration 2 U/μl, Merck, Germany) according to the manufacturer's instructions. After incubation, enzymes were inactivated by heating and used at further experiments.

To determine the effects of eDNA and gDNA on biofilm establishment of *S.* Typhimurium DMC4 and *S.* Typhimurium SL1344, strains were incubated with enzyme-treated and un-treated DNA for biofilm production. Biofilm formation experiments were performed as described above. Additionally, 10 μl from each DNA sample was mixed with 190 μl of OD595=0,2 adjusted *Salmonella* strains and incubated at 20 °C for 12, 24 and 48 hours. At least six wells of the 96-well microdilution plate inoculated with test strain only, to test biofilm production. End of the incubation period, as detailly described above unattached bacteria were removed, biofilm structure was stained and optical densities (OD$_{595}$) of biofilm structure were measured. The results were obtained by comparing results of the median OD$_{595}$ of the triplicates of the control (biofilm formed with the presence of un-treated enzyme) from the median OD$_{595}$ of the triplicates of the sample (biofilm formed with the presence of nuclease-treated enzyme) **(Tetz et al., 2009)**.

Statistical Analysis

R statistical software was used to perform the statistical analysis. The accuracy of the data obtained as a result of our experiments was determined by the ANOVA test. The data evaluated as "meaningful" or "meaningless" according to the calculated F value by the end of variance analysis. Evaluations between meaningful groups were carried out by Tukey's Accuracy Test **(Neupane et al., 2014)**.

RESULTS

Quantification of eDNA Concentration in Planktonic and Biofilm Forms of *S.* Typhimurium Strains

eDNA presence was quantitatively determined in planktonic cultures and biofilm forms of *Salmonella* strains by using PicoGreen® labeling. As a result, it was determined that planktonic cultures of *S.* Typhimurium SL1344 and DMC4 strains produced 313,18 ng/μl and 319,37 ng/ μl eDNA, respectively (Figure 1). Among three different incubation periods, maximum eDNA isolated from planktonic cells following the incubation at 20 °C for 24 hours.

In biofilm forms of these bacteria, similar to planktonic forms, maximum eDNA concentration was detected at 20 °C following the incubation for 24 hours. It was determined that 24 hour *S.* Typhimurium SL1344 and DMC4 biofilms contained 206 ng/μl and 211 ng/μl eDNA, respectively (Figures 2 and 3). eDNA content of biofilm structure is less than planktonic counterparts of the same strains.
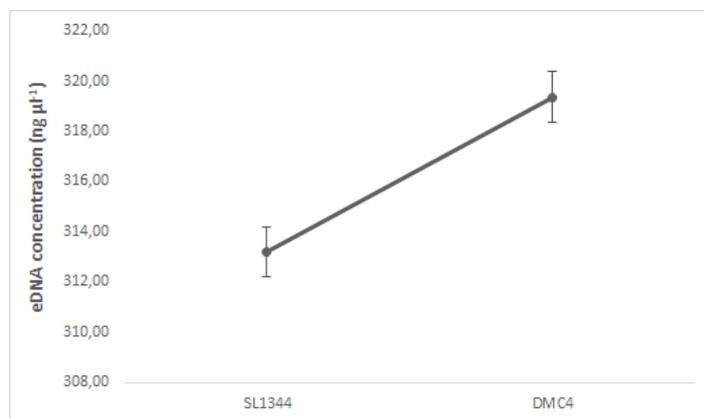

Figure 1 eDNA concentrations, isolated from planktonic forms of *S.* Typhimurium DMC4 and SL1344
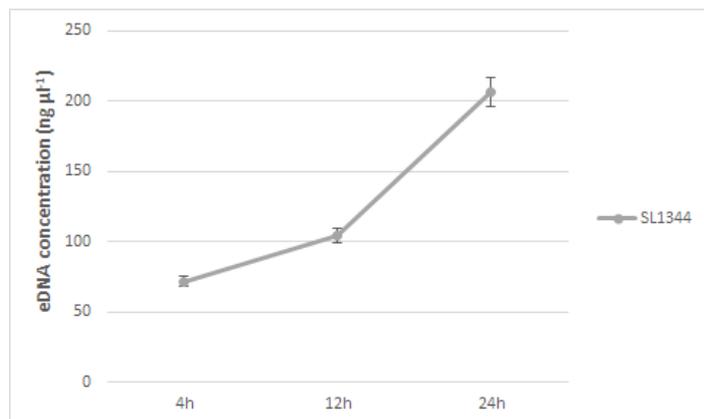

Figure 2 eDNA concentrations isolated from biofilm forms of *S.* Typhimurium SL1344
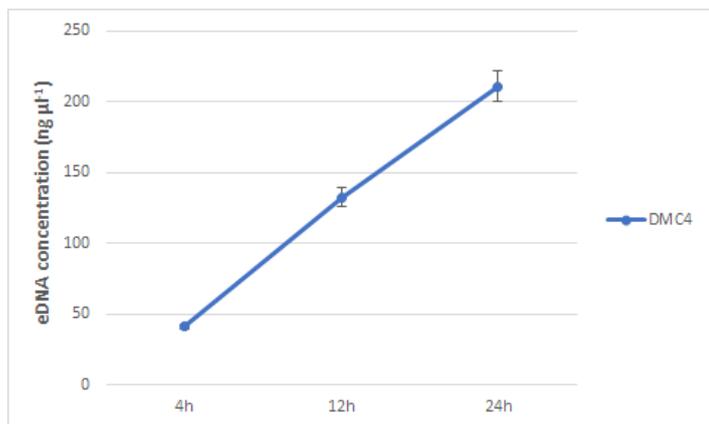
Figure 3 eDNA concentrations isolated from biofilm forms of *S*. Typhimurium DMC4

The Effects of eDNA and gDNA on the establishment of biofilm formation of *Salmonella* Strains

Three different eDNA and gDNA concentrations (20, 50 ve 100 µg/mL) were tested in the preliminary experiments conducted to determine the efficacy of exogenous DNA on biofilm establishment of *Salmonella* strains. Since the highest enzyme activity was determined at 100 µg/mL of both DNaseI and Benzonase® (data not shown), further experiments were conducted using 100 µg/mL of eDNA and gDNA. Two different control groups were constructed: (i)Positive Control (PC) group, which expresses the biofilm structure in the presence of enzyme-free nucleic acids, (ii) Negative control (NC) group, which expresses biofilm without external nucleic acid addition. Examination of biofilm structures developed in the presence of enzyme-treated nucleic acids of *S*. Typhimurium strains suggests that statistical evaluations in terms of the resulting data are significant ($p < 0.01$)

For both tested *Salmonella* strains, it was determined that the biofilms developed in the presence of DNase I and Benzonase®-treated eDNA and gDNA samples were found to be higher in statistically significant levels ($p < 0.01$) than the biofilms developed by control groups (Figure 4). However, this effect, which was determined at the end of 24 hours on biofilm structures, reversed after 48 hours and significant decreases ($p < 0.01$) determined in biofilm production in the presence of DNaseI and Benzonase® compared to the control groups (Figure 5).
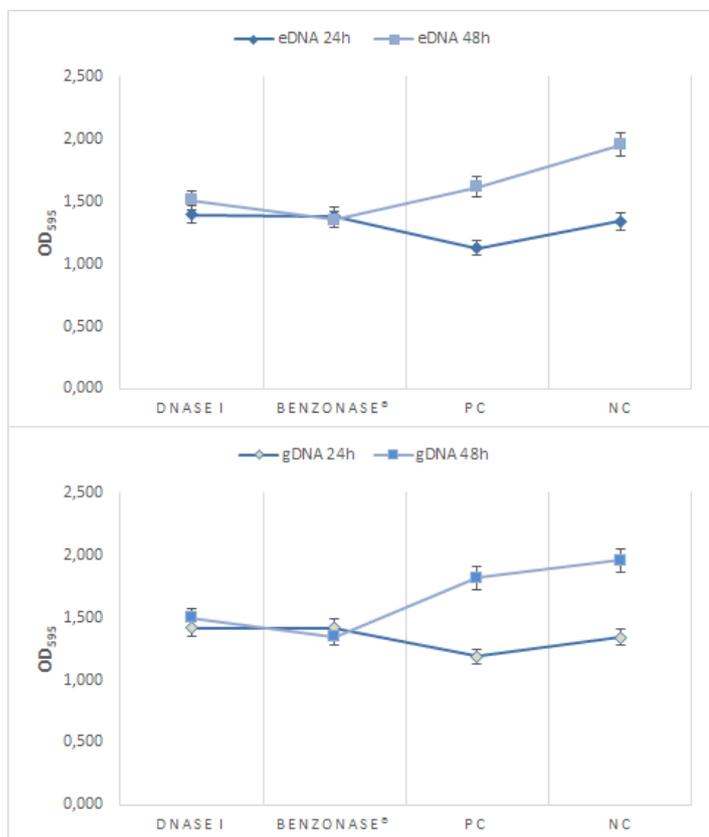

Figure 4 The effect of eDNA and gDNA on the SL1344 biofilm production at 24h and 48h. DNase I: Biofilm formation in the presence of nucleic acids with adding 100 µg/mL DNase I. Benzonase®: Biofilm formation in the presence of

nucleic acids with adding 100 µg/mL Benzonase®. PC: Biofilm formation in the presence of nucleic acids without enzyme addition. NC: Biofilm formation without nucleic acid addition.

Figure 5 The effect of eDNA and gDNA on the DMC4 biofilm production at 24 h and 48 h. DNase I: Biofilm formation in the presence of nucleic acids with adding 100 µg/mL DNase I. Benzonase®: Biofilm formation in the presence of nucleic acids with adding 100 µg/mL Benzonase®. PC: Biofilm formation in the presence of nucleic acids without enzyme addition. NC: Biofilm formation without nucleic acid addition.
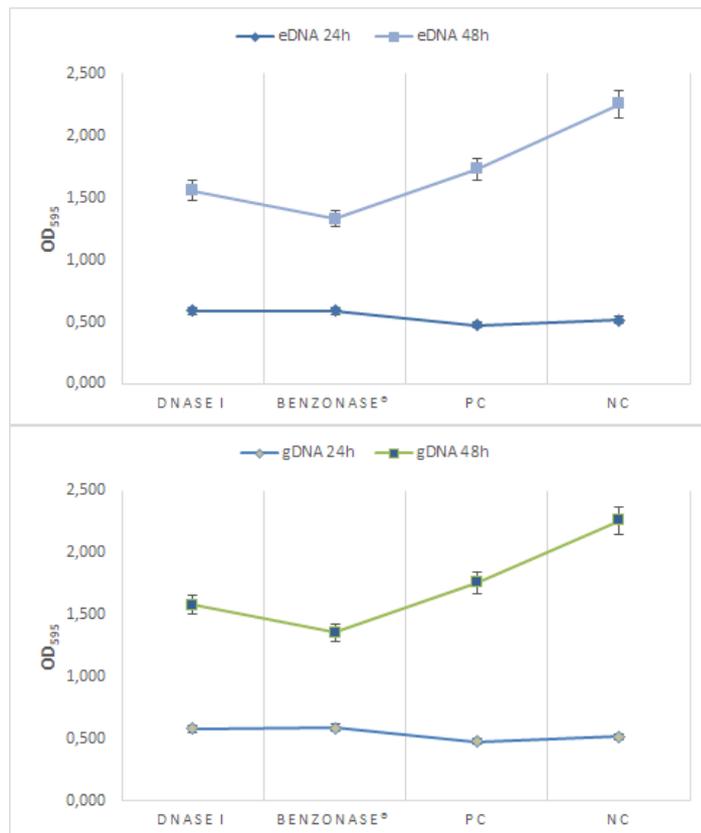
DISCUSSION

The fact that eDNA, the major component of microbial biofilm structures, is a potential target for inhibition or eradication of the formation of biofilm structures, particularly in the medical and food industries, has intensified efforts to determine the function of eDNA in biofilm formation. It was determined that eDNA has key functions in the adhesion, maturation and structural organization of biofilms containing single species of bacteria such as *Listeria*, *Staphylococcus*, *Microbacterium*, *Xanthomonas*, *Serratia* and *Pseudomonas* and multi species-containing biofilms such as dental biofilms or mixed biofilms formed by *Staphylococcus epidermidis* and *Candida albicans* (**Harmsen *et al.*, 2010; Pammi *et al.*, 2013; Sena-Vélez *et al.*, 2016; Shopova *et al.*, 2013; Sugimoto *et al.*, 2018; Tang *et al.*, 2013; Vorkapic *et al.* 2016**). However, it has also found that eDNA plays an inhibitory role in binding and maturation of biofilm in *S*. Typhi and *S*. Typhimurium and *Claulobacter crescents* (**Berne *et al.*, 2010; Wang *et al.*, 2014**). These conflicting findings on the role of eDNA in the biofilm formation require that the studies be continued in detail.

In this study, the role of eDNA and gDNA on biofilm structures of *S*. Typhimurium SL1344 and DMC4 strains was monitored for 24 and 48 hours. As a result of both experiments, it has been found that both eDNA and gDNA from these strains play an inhibiting role in biofilm formation at the initial stage. However, between 24-48 hours, the addition of eDNA and gDNA contributed to the formation of biofilm. In the case of 24 hour samples, administration of DNase I affected the formation of biofilm positively and showed an inhibitory effect on biofilm formation between 24-48 hours. The detection of more eDNA in planktonic cells of both of the tested strains than the first 24 h of biofilm indicates that eDNA is not a specific component of biofilm and has some basic functions in planktonic forms. The fact that both eDNA and gDNA have similar effects on biofilm structures confirms this predicament. Amyloid protein, one of the major components of *Salmonella* biofilms, is known to form highly immunogenic composite structures by tightly interfering with curli fimbriae and DNA in the structure. It is known that these composites cause an immune response in the host organism and activate the production of autoantibodies (**Gallo *et al.*, 2015; Oppong *et al.*, 2015**). In addition to these properties, amyloid-DNA complexes have also been shown to improve the structural

stability of biofilms **(Spaulding *et al.*, 2015)**. For example; In *Staphylococcus aureus*, it has been found that eDNA plays a key role in the formation of amyloid fibers in the biofilm structure and that the polymerization of amyloid fibers does not occur if the eDNA is not in the biofilm matrix structure **(Taglialegna *et al.*, 2015)**.

As summarized above, the different effects of eDNA on biofilm formation and stages in different bacterial species are because of eDNA does not exhibit the same interactions as the biofilm matrix material differs among bacterial species. The results of our study indicate that the tight association of extracellular DNA with curly fibers in the early stages of biofilm formation in *Salmonella* suggests that curly fibers that play a major role in adhesion degrade this activity and in part cause the inhibition of early stages of biofilm. However, after biofilm attachment and colonization, DNA-curli fiber composites stabilize the biofilm matrix and contribute to biofilm stability. Further molecular analysis should be performed to support our findings. Another important finding in our study is that the origin of DNA in the biofilm matrix has the same effect on biofilm formation. This supports our predictions, which describe the activity of curli fibers and DNA complexes on *Salmonella* biofilms.

CONCLUSION

In our study, it is important to determine that DNA samples (eDNA and gDNA) inhibit the early stages of biofilm formation in *S*. Typhimurium independently from its source, which is of great importance for industrial and medical studies aiming to prevent biofilm formation in *Salmonella* at the early production stages. Chemical and biological agents (especially lytic enzymes and viruses) that accelerate the release of DNA appear to have significant potential in this sense, considering both the ability to break down cells that will form biofilms and to reduce the effectiveness of biofilm formation of lytic enzymes and virus resistant cells. On the other hand, determining the production of eDNA in the planktonic forms of *S*. Typhimurium at higher levels than the biofilm forms disproves the view that eDNA is produced in the biofilm process and suggests that eDNA has more important functions in planktonic forms than biofilm forms. Further genetic and functional studies are needed to clarify this condition.

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