



Effects of sub-MIC antibiotic concentrations on biofilm production of *Salmonella* *Infantis*

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ABSTRACT

In the present study, 13 *Salmonella* *Infantis* strains, which have been originated from Turkey, were selected due to their clinical and industrial relevance, sufficient biofilm producing capability and multidrug resistance. Although all tested strains were built up of thin pellicle, optimum pellicle formation has occurred at 28 °C. All *S. Infantis* biofilms were categorized as 'bdar' morphotype following the incubation at both 20 and 28 °C, while they were categorized as 'saw' morphotype at 37 °C. Under a certain incubation temperature (28 °C), 84.62% of strains have formed strong biofilm structures. By using the disk diffusion method, high levels of resistance have been observed among tested bacteria against nalidixic acid (100%), spectinomycin (100%), streptomycin (92.3%), tetracycline (92.3%), kanamycin (76.9%) and neomycin (76.9%). Further studies were performed with *S. Infantis* DMC 12 strain, due to its capability to produce biofilm and multidrug resistance phenotype. Gentamycin (>64 µg/mL, 2 × MIC) and tetracycline (>128 µg/mL, 4 × MIC) were determined as the most effective antibiotics against biofilm formation. The biofilm forms have showed increased antimicrobial resistance when it was compared to the planktonic bacteria. The highest resistance rates of the biofilm bacteria were observed to neomycin (12 × MIC) followed by spectinomycin (10 × MIC) and streptomycin (10 × MIC). Biofilm structure was induced as a result of nalidixic acid, spectinomycin, tetracycline and neomycin treatment at sub-MIC concentrations of tested antibiotics.

ARTICLE HISTORY

Received 21 January 2016

Accepted 12 August 2016

KEYWORDS

Salmonella *Infantis*; antibiotic resistance; biofilm; induction; MIC; sub-MIC

Introduction

Salmonella species are enteric pathogens and the leading cause of bacterial foodborne illness. *Salmonella* species can form multicellular structures, called biofilms, on various surfaces such as polymer, stainless steel and glass, as well as biotic surfaces such as gallstones and epithelial cells [1,2]. *Salmonella* may also form biofilms (pellicles) in the air–liquid interphase [3].

Bacterial biofilms are described as bacterial communities that can be attached to abiotic or biotic surfaces and enclosed with self-produced extracellular matrix substances [1]. Generally, the bacterial cells in biofilm, embedded within a matrix structure, are well protected due to a chemical and physical barrier against various undesirable environmental conditions.

Around the world, antibiotic resistance in *Salmonella* sp. is a re-emerging problem. Determination of the antibiotic resistance profiles of *Salmonella* sp. from different serovars is important in order to apply suitable treatment against *Salmonella* infection. Biofilms are resistant to eradication by antibiotics at concentrations that are

10–1000 times greater than concentrations needed to eradicate free-living cells [4]. Though sub-minimal inhibitory concentrations (sub-MIC) of some antibiotics can inhibit biofilm formation, it is known that they are not able to kill bacteria. On the other hand, numerous studies have shown that, when present at concentrations below the MIC, some antibiotics can significantly induce biofilm formation in a variety of bacterial species *in vitro*.

In the present study, antibiotics used for the treatment of *Salmonella* infections were tested against *Salmonella* *Infantis* serovar, including biofilm formation, morphotype determination, and we also determined the biofilm inhibition or induction at sub-MICs of tested antibiotics. For the first time, this report shows that certain antibiotics induce biofilm development of *S. Infantis* serovar.

Materials and methods

Salmonella *Infantis* isolates

The 13 DMC coded (DMC 7, 12, 20, 23, 40, 41, 57, 58, 70, 71, 75, 76, 77) *Salmonella* *Infantis* strains, isolated from

raw chicken samples collected from Turkey, were all obtained from Prokaryotes Genetics Laboratory, Biology Department, Ankara University. All isolates were stored at -80°C in a glycerine freezing medium and recovered in Luria Bertani (LB; Merck KGaA, Germany) at 37°C overnight. LB without NaCl ($\text{LB}^{\text{wo}}/\text{NaCl}$; bacto-tryptone 10 g/L, yeast extract 5 g/L) was used as a test broth in the biofilm assays [5,6].

Screening of biofilm morphotype

All 13 *S. Infantis* strains were screened at least three times for colony morphology by using the method described previously [6]. Briefly, colonies were inoculated onto LB agar without NaCl containing 40 $\mu\text{g}/\text{mL}$ Congo red (Sigma-Aldrich, Germany). The CR agar plates were incubated at 20, 28 and 37°C , and were examined using dissection microscope (Leica, Germany) under $1\times$ magnification. Isolates were grouped into three distinct morphotypes: (1) red, dry and rough, indicating curli and cellulose production (rdar); (2) brown, dry and rough, indicating curli production but a lack of cellulose synthesis (bdar); and (3) smooth and white, indicating a lack of both curli and cellulose production (saw).

Quantification of biofilm formation

Biofilm formation was quantified in $\text{LB}^{\text{wo}}/\text{NaCl}$ as previously described [6]. Briefly, cells were cultured overnight in the appropriate medium and then diluted in $\text{LB}^{\text{wo}}/\text{NaCl}$ to $\text{OD}_{595} = 0.2$ ($\sim 10^8$ CFU/mL) (Shimadzu, Japan) and inoculated into 96-well polystyrene microtitre plates. Plates were incubated overnight at 20, 28 and 37°C under static conditions, and were gently washed twice with sterile physiologic sodium chloride solution (0.85% NaCl). The plates were dried in room temperature before adding 130 μL 33% glacial acetic acid (v/v) (Sigma-Aldrich, Germany) and incubated for 30 min in room temperature. Following the incubation, solubilized dye from the adherent cells was measured at OD_{595} . For each strain, the result was calculated by subtracting the median OD_{595} of the three parallels of the control (test broth only) from the median of OD_{595} of the three parallels of sample. The cut-off value (OD_c) for a biofilm producer and the classification of the isolates as non-biofilm producers were determined as ($\text{OD} \leq \text{OD}_c$), for weak biofilm producers ($\text{OD}_c < \text{OD} \leq 2 \times \text{OD}_c$), for moderate biofilm producers ($2 \times \text{OD}_c < \text{OD} \leq 4 \times \text{OD}_c$), and for strong biofilm producers ($4 \times \text{OD}_c$) [7].

Biofilm formation in liquid–air interface

To analyse biofilm formation in liquid, i.e. pellicle formation at the liquid–air interface, 4.5 mL of $\text{LB}^{\text{wo}}/\text{NaCl}$ was

inoculated with 0.5 mL of an overnight culture and incubated at 20, 28 and 37°C for eight days. At the end of the incubation process, the strains were also evaluated by their appearance, to be able to see if they showed elastic brittle and rigid features [3].

Antibiotic susceptibility testing

The following antimicrobial agents were selected due to their common use for the treatment of *Salmonella* infections. Therefore, the study evaluated the bacterial resistance against kanamycin (30 μg), neomycin (10 μg), nalidixic acid (30 μg), tetracycline (30 μg), spectinomycin (10 μg), trimethoprim (5 μg), ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), ceftiofur (30 μg), chloramphenicol (30 μg), florfenicol (30 μg), gentamicin (10 μg), ciprofloxacin (5 μg), trimethoprim/sulfamethoxazole (25 μg) and streptomycin (10 μg). All solutions were sterilized by using a 0.2 μm cellulose syringe filter (Sartorius, Germany). Agar disk diffusion and MIC were performed using Mueller-Hinton agar (MH-Agar) and Mueller-Hinton Broth (MHB, Oxoid), respectively, according to CLSI guidelines [8]. *Escherichia coli* ATCC 25922 strain was used as a control.

Effect of sub-inhibitory concentrations (sub-MICs) of tested antibiotics on biofilm formation

The activities of antimicrobial agents were tested at a concentration ranged from 0.5 to 512 $\mu\text{g}/\text{mL}$ to determine their effects on biofilm formation. Various concentrations of antibiotics, prepared in 200 μL of MHB, were added to wells of 96-flat bottom plates containing 20 μL of diluted ($\text{OD}_{595} = 0.2$) overnight culture of bacteria. After incubation at 28°C for 24 h, quantification of biofilm was measured as previously described and control wells contained only drug-free medium [9].

Effects of antimicrobial agents on the reduction of Salmonella biofilms on stainless steel coupons

Stainless-steel-type 304 ($1 \times 8 \times 9 \text{ mm}^3$) coupons were cleaned with 100% acetone, dried and cleaned with 70% alcohol (v/v). Cleaned coupons were autoclaved at 121°C for 15 min. Previously proposed biofilm formation model was used to develop biofilm structure on stainless steel coupons and they were individually placed in sterile tubes that contain 3.5 mL of $\text{LB}^{\text{wo}}/\text{NaCl}$ and 1 mL of overnight culture, adjusted to 10^8 CFU/mL, and then incubated at 28°C for 24 h [10]. Following the incubation, coupons were removed with sterile forceps, washed with sterile physiologic sodium chloride solution (to remove loosely attached cells), transferred to a sterile tube and air dried for 10 min. Subsequently, coupons

were individually transferred into tubes containing 2 mL of various antibiotic concentrations with a serial twofold dilution of each antibiotic; (MIC/2 to MIC/1024 µg/mL) and incubated at 37 °C for 24 h. The positive control tubes only contained MHB. After incubation, stainless steel coupons were removed, washed and dried as described above. Five sterile glass beads ($r = 3$ mm) and dried coupons were placed in a 10-mL tube which contained 4.5 mL of sterile physiologic sodium chloride solution and tubes were vortexed for 1 min. CFU counts were enumerated by spread plating in LB agar and incubated at 37 °C for 24 h. Antibiotic concentrations eradicate 99.9% of biofilm structure compared to control group was determined as Minimum Biofilm Eradication Concentration (MBEC). MBEC experiments were performed by using multidrug-resistant and biofilm producer *S. Infantis* DMC 12 strain.

Statistical analysis

All statistical analyses were performed by using SPSS (version 18, USA). The assays for inactivation of performed biofilms were replicated three times independently. One-way ANOVA test was preferred for microtitre plate assay data and MBEC data.

Results and discussion

Even though *S. Infantis* serovar, which was used in the present study, is not the common serovar in *Salmonella* epidemiology, it is one of the most frequently isolated serovar from clinical and food environment. Because of the widespread usage of antimicrobial agents in both developed and developing countries, antibiotic resistance in some types of *Salmonella* infections is increasing. Increasing antimicrobial resistance in non-typhoid *Salmonella* species has been a serious problem for public health [11,12]. Antibiotic resistance profiles of food-originated DMC coded 13 *S. Infantis* isolates, determined in the present study are compatible with the previous findings [13–15]. The most prevalent resistances, found in the multidrug-resistant DMC coded *S. Infantis* strains, were to nalidixic acid (100%) and spectinomycin (100%). Previous reports indicated the potential emergence of quinolone resistance in *Salmonella* [14,16–18]. It is known that isolates with nalidixic acid resistance have commonly decreased susceptibility to ciprofloxacin. Similar to this knowledge, as a result of antimicrobial susceptibility assays obtained from the present study, ciprofloxacin resistance was determined in 76.9% of tested *S. Infantis* isolates.

A total of 13 isolates studied, 10 (76.9%) were sensitive to ciprofloxacin, 1 (7.69%) was sensitive to

Table 1. MIC values of tested *S. Infantis* strains.

Strain code	GEN*	KAN*	NAL*	NEO*	SPE*	STR*	TET*
DMC 7	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 12	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 20	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 23	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 40	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 41	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 57	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 58	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 70	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 71	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 75	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 76	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)
DMC 77	R (≥ 32)	R (≥ 64)	R (≥ 128)	R (≥ 32)	R (≥ 128)	R (≥ 32)	R (≥ 32)

*GEN, gentamycin; KAN, kanamycin; NAL, nalidixic acid; NEO, neomycin; SPE, spectinomycin; STR, streptomycin; TET, tetracycline; R, resistant

Table 2. MBEC values of tested *S. Infantis* strains.

Antibiotics	MIC values (µg/mL)	MBEC values (µg/mL)
Gentamycin	>32	>64 (2 × MIC)
Kanamycin	>64	>512 (8 × MIC)
Nalidixic acid	>128	>1024 (8 × MIC)
Neomycin	>32	>384 (12 × MIC)
Spectinomycin	>128	>1280 (10 × MIC)
Streptomycin	>32	>320 (10 × MIC)
Tetracycline	>32	>128 (4 × MIC)

tetracycline. By using the disk diffusion method, high levels of resistance among tested bacteria were observed against nalidixic acid (100%), spectinomycin (100%), streptomycin (92.3%), tetracycline (92.3%), kanamycin (76.9%) and neomycin (76.9%). The concentrations of antibiotic that were required to inhibit planktonic (MIC) and kill biofilm bacteria (MBEC) are summarized in Tables 1 and 2. Among seven tested antibiotics, the highest resistance was observed against nalidixic acid (≥ 128) and spectinomycin (≥ 128), followed by kanamycin (≥ 64). The incidence of multidrug resistance (MDR to three or more antibiotics) in *Salmonella* strains was 100%. All MBEC levels were determined higher than the MIC values. The most effective antibiotic, against biofilm formation, was gentamycin (>64 µg/mL, 2 × MIC), followed by tetracycline (>128 µg/mL, 4 × MIC).

World Health Organization has classified quinolones as critically important antimicrobial agents for human medicine. Unfortunately, the incidence of resistance to quinolones in bacteria from food-producing animals has been recognized as a primary public health problem [19]. Quinolones and fluoroquinolones are broad-spectrum antimicrobial agents that target DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV genes (*parE* and *parC*), and the resulting drug–enzyme–DNA complex inhibits DNA synthesis [13]. Mobile genetic elements such as plasmids, transposons, and the more recently explored integrons, which are able to disseminate antibiotic resistance genes by horizontal or vertical transfer, have played an important role in the evolution and dissemination of MDR in Gram-negative bacteria [20,21]. Further investigations are required to identify the genetic nature of antibiotic resistance of the 13 *S. Infantis* strains.

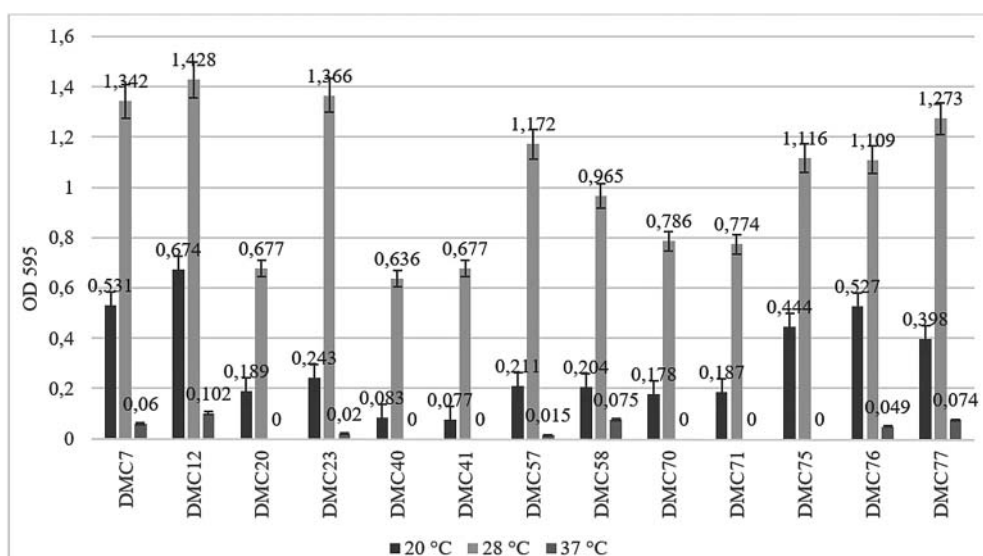


Figure 1. Biofilm production levels of DMC coded 13 *S. Infantis* strains following the incubation at 20, 28 and 37 °C on polystyrene plates.

A biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix which consists of polysaccharide, protein and DNA. Bacteria in biofilms are known to be more resistant to antimicrobials and drying [22]. Curli fimbriae, proteinaceous extracellular fibres and cellulose are major components of the extracellular matrix of *Salmonella typhimurium* biofilms [5,23,24]. It was reported that the contribution of the extracellular matrix was variable in environmental conditions [24]. In the present study, DMC coded 13 *Salmonella* isolates displayed two different biofilm morphotypes; 'bdar' morphotype after incubation at 20 and 28 °C, 'saw' morphotype after incubation at 37 °C in LB^{wo}/NaCl supplemented with Congo Red (40 µg/mL).

In the screen of food-originated 13 *Salmonella* strains, OD₅₉₅ values were found to range from 0.07 to 0.67 at 20 °C, 0.67 to 1.42 at 28 °C and 0 to 0.1 at 37 °C (Figure 1). Statistically significant differences between serovars were observed as a result of one-way ANOVA, $p < 0.01$. Since the highest intensity of biofilm formation on polystyrene was produced by DMC 12 coded *Salmonella* Infantis strain at 28 °C, further analyses were performed by using DMC 12. At 20 and 28 °C, 24.8% and 76.9% of *S. Infantis* strains produced strong biofilm, respectively. Also, all strains were tested for their biofilm-forming abilities at the liquid–air interface. Although all tested strains were built up of thin pellicle after growth at 20, 28 and 37 °C, maximum pellicle formation occurred at 28 °C (Figure 2).

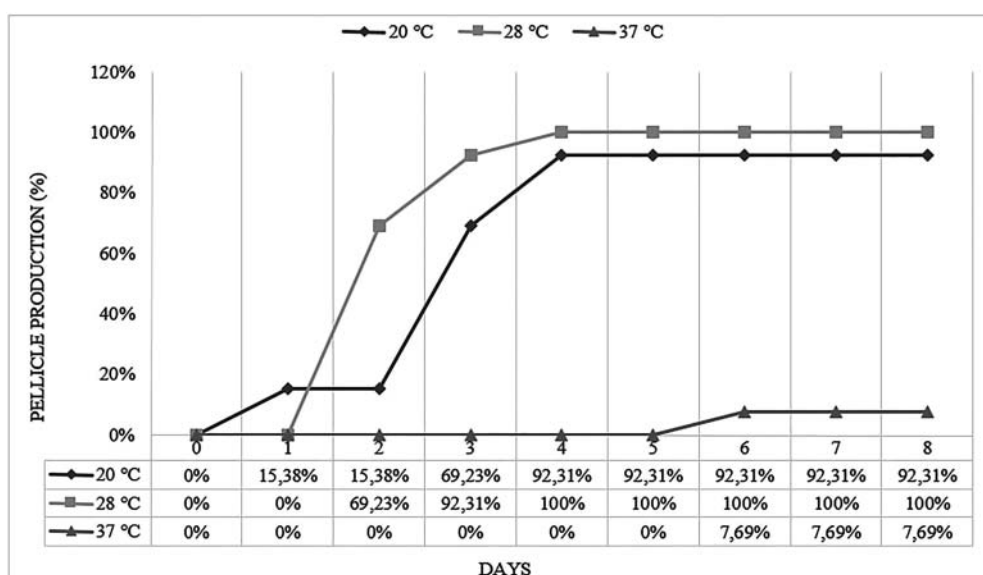


Figure 2. Pellicle production levels of *S. Infantis* strains at 20, 28 and 37 °C for eight days.

Researchers reported that the 'bdar' morphotype is rarer in natural isolates and lacking only the cellulose component of rdar morphotypes [6]. The bdar morphotype has been suggested to be linked to *Salmonella* groups with a narrower host range that may not need to survive for long periods in the environment and are potential commensals with their hosts [22,25]. As it was previously mentioned, *S.* serovar is not the common serovar in *Salmonella* epidemiology and all tested isolates formed 'bdar' morphotype at 20 and 28 °C, our findings match with the previous reports [22,25].

Curli and cellulose production behaviour of 51 *Salmonella typhimurium* strains in different conditions of the biofilm formation has been analysed [24]. They concluded that biofilm forming behaviour is not only affected by environmental conditions such as temperature and the culture medium, but also it was associated with strain origin. Steponovic et al. [26] reported that 1.6% of the tested 122 *Salmonella* strains produced strong biofilm on polystyrene at 28 °C [26]. De Oliveira et al. [27] also assessed biofilm production abilities of 171 *Salmonella* spp. on stainless steel, glass and PVC (the most similar to polystyrene material) [27]. They observed that 1.7% of strains growth on stainless steel and glass, but PVC was not colonized at 28 °C. In our previous study, we tested biofilm-producing abilities of *Salmonella* strains at different environmental conditions such as temperature, pH and NaCl concentrations [28]. The highest amount of biofilm was formed by all tested strains on polystyrene at 20 °C, and it was produced at similar levels by *S. typhimurium* (rdar, OD₅₉₅ = 3.481) and *S. Infantis* (bdar, OD₅₉₅ = 3.478) strains. Biofilm contents and physiology are highly heterogeneous and varies depending on the material of the surface, growth conditions, environmental conditions and gene expression levels that are affected by these factors. It can be concluded that, since suitable conditions and gene expressions occurred at 28 °C, we determined optimum production for *S. Infantis* serovar at this incubation temperature.

Bacteria protected within biofilms are 10–1000 times more resistant to antibiotics than planktonic cells [4]. There are numerous factors such as contact time, the surrounding environment and the concentration of the antimicrobial agent to determine its effectiveness. Although MIC has been used as a gold standard to determine antimicrobial sensitivities for bacteria, the MIC value is not predictive of clinical efficacy of a particular antibiotic [29]. The MBEC assay was developed for rapid and reproducible antimicrobial susceptibility testing for bacterial biofilms. It has been reported that MBEC values are greater than MIC against isolated strains from *Salmonella* infections [30,31]. In the present study, at first, we

determined the MBEC levels of seven different antibiotics on *S. Infantis* serovar biofilms. Also, few publications are available on MBEC values of several antibiotics on other *Salmonella* serovars. Olson et al. [29] determined antibiotic sensitivities of certain bacteria including *Salmonella* Bredeney, *S. typhimurium* and *Salmonella* sp. [29]. Parallel to our findings, there was considerable variability among the MICs of the *Salmonella* spp. isolates; similar variability between the MIC and MBEC values was observed. Besides *Salmonella* strains, they also tested other Gram-negative livestock pathogens such as bovine, porcine and avian *Pasteurella* spp., as well as the *Haemophilus somnus*, and *Mannheimia haemolytica*, formed biofilms, but in most cases, there was not any difference between the MIC and MBEC values. Papavasileiou et al. [32] compared antimicrobial susceptibility of biofilm versus planktonic forms of 194 *Salmonella enterica* strains isolated from children gastroenteritis [32]. The biofilm forms showed increased antimicrobial resistance compared to the planktonic bacteria. The highest resistance rates of the biofilm bacteria were observed with respect to gentamicin (89.9%) and ampicillin (84.4%). *S. enterica* strains isolated from children with gastroenteritis. Combining our results with the limited previous reports, this observations reflected the complexity in prediction of chemotherapeutic agents for treatment of different *Salmonella* isolates, since higher dosage of antibiotic requires to combat with biofilm structure.

The activities of antimicrobial agents tested at sub-MIC concentrations against DMC 12 *S. Infantis* biofilm are shown in Figure 3. Gentamycin, streptomycin and kanamycin significantly ($p < 0.05$) reduced optical density of the biofilm of DMC 12. Gentamycin (MIC ≤ 2) was the most effective antibiotic in eradication of bacterial biofilm structure. The exposure of bacteria to nalidixic acid, spectinomycin, tetracycline and neomycin induced the biofilm production ($p < 0.05$) (Figure 3).

As reviewed by Kaplan [33], although numerous studies have shown that sub-MICs of some antibiotics are not able to kill bacteria, but they can inhibit biofilm formation [33]. In contrast, many studies have shown that some antibiotics, when present at concentrations below the MIC, can significantly induce biofilm formation in variety of bacterial species *in vitro*. The results of our study, as presented in Figure 3, show that when *S. Infantis* strain was grown in the presence of sub-MIC of antibiotics, the maximum inhibition of biofilm formation was observed as a result of gentamycin treatment. The exposure of bacteria to nalidixic acid, spectinomycin, tetracycline and neomycin induced the biofilm production ($p < 0.05$). As it was performed in the previous study, sub-MIC concentrations of tobramycin-induced *Pseudomonas aeruginosa* biofilm formation through a mechanism that

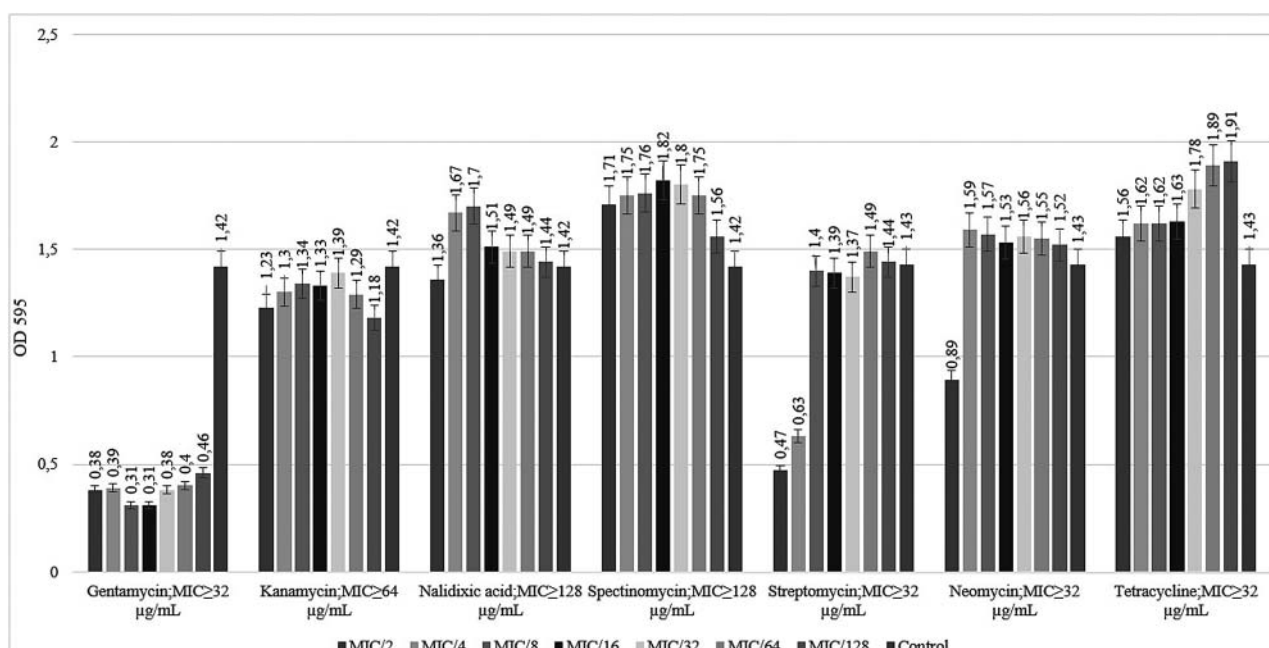


Figure 3. Effect of sub-MIC of seven tested antibiotics on biofilm formation of *S. Infantis* DMC 12.

involves the intracellular second messenger cyclic dimeric guanosine monophosphate (c-di-GMP) [34]. Other researchers have studied the modulation of *Salmonella* gene expression by sub-MIC concentrations of quinolones [35]. Even at the sub-MICs employed, all tested fluoroquinolones upregulated genes involved in the SOS response, *umuD*, *LexA*, *sbmC* and *dinP*. They concluded that small molecules can have functions other than growing the inhibition that may affect the establishment and maintenance of community dynamics in complex environments. Wojnicz and Tichaczek-Goska [9] analysed the effect of sub-MIC of ciprofloxacin, amikacin and colistin on biofilm formation of *E. coli* isolates [9]. Their results showed that all three tested antibiotics, that reduced biofilm production, interfere with curli expression but do not influence motility. They suggested that ciprofloxacin, amikacin and colistin may be useful in the treatment of biofilm-associated infections caused by *E. coli* strains. As a result of our study, at first, we analysed the effect of sub-MICs of seven antibiotics on biofilm formation of *S. Infantis* serovar. Consequently, further investigations are required to be able to determine molecular mechanisms that involve the inhibition of *S. Infantis* biofilms at sub-MIC concentrations.

Conclusion

In conclusion, the present study is the first report that assessed antibiotic susceptibility, besides MBEC and sub-MICs of tested antibiotics on *S. Infantis* biofilm structure. This study showed the diversity of response to antibiotic treatment in *S. Infantis* serovars depending on their cell

forms, planktonic cells or biofilms. Although *S. Infantis* serovars were sensitive to gentamycin antibiotic while they formed biofilm, they showed much greater resistance to nalidixic acid in planktonic cultures. This may be due to the same bacterium which is different in point of both genetic and physiologic in the biofilm state than in the planktonic state for which the antibiotic was designed and tested. It is concluded that biofilm-forming *S. Infantis* bacteria are able to escape *in vitro* the action of commonly used antibiotics. Kaplan reviewed that 'this process may have clinical relevance because bacteria are exposed to sub-MIC concentrations of antibiotics at the beginning and the end of a dosing regimen, between doses, or continuously during low-dose therapy' [33,36,37]. Knowledge about the consequences of the usage of antimicrobial agents at treatment of *Salmonella* infections is required to be examined and needs to be decided the concentrations considering biofilm structure.

Acknowledgement

We thank Cihan Akın GÜRSOY for proofreading and editing our research paper.

Disclosure statement

No potential conflict of interest was reported by the authors.

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