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DETERMINATION OF ANTIMICROBIAL EFFECT OF THE AQUEOUS EXTRACT OF STINGING NETTLE (*URTICA DIOICA*) ON BIOFILM FORMATION OF *SALMONELLA ENTERICA* SEROVARS

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ABSTRACT

Due to health concerns associated with the increase of antimicrobial resistance in foodborne pathogens such as *Salmonella*, plant extracts have become important natural alternatives to known antimicrobials. The eradication effect of the water-soluble stinging nettle extracts with 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL, and 20 mg/mL concentrations, on pre-formed biofilms and swimming motility of *Salmonella enterica* subspecies *enterica* serovars, including Newport, Typhimurium, Enteritidis, Virchow, Othmarschen and Mikawasima, was investigated *in vitro*. Degradation of biofilm formation on spinach inoculated with each serovar was ascertained within different exposure time of 40 mg/mL concentrated extract as well. Moreover, major genes responsible for biofilm formation (i.e., *rpoS, mlrA, ycf*R, *fimA, spiA* and *csgA*) were screened in these isolates. The extract significantly decreased swimming motilities of Mikawasima and Virchow serovars. The highest reductions were found as 0.88 Log CFU/mL and 2.00 Log CFU/cm^2 *in vitro* and on spinach, respectively.
Key words: Antimicrobial effect, biofilm, *Salmonella*, stinging nettle, swimming motility

ISIRGAN OTU (*URTICA DIOICA*) SULU EKSTRAKTININ *SALMONELLA ENTERICA* SEROVARLARININ BİYOFİLM OLUŞUMU ÜZERİNE ANTİMİKROBİYEL ETKİSİNİN BELİRLENMESİ

ÖZ

Salmonella gibi gıda kaynaklı patojenlerde antimikrobiyel direncin artması yol açacağı sağlık problemleri nedeniyle endişelendirmektedir. Bu bağlamda, bitki özleri bilinen antimikrobiyellere önemli doğal alternatifler haline gelmiştir. Suda çözünür ısırgan otu ekstraktlarının 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL ve 20 mg/mL konsantrasyonları ile eradikasyon etkisi, Newport, Typhimurium, Enteritidis, Virchow, Othmarschen ve Mikawasima dahil olmak üzere *Salmonella enterica* alt türü *enterica* serovarlarının önceden oluşturulmuş biyofilmleri ve yüzme hareketliliği in vitro olarak araştırılmıştır. Her serovar ile bulaştırılan ıspanakta, izolatların oluşturduğu biyofilm oluşumunun engellenmesi için 40 mg/mL konsantre ekstraktın etkin olduğu tespit edilmiştir.

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Ayrıca, biyofilm oluşumundan sorumlu ana genler (yani *rpoS, mlrA, ycf*R*, fimA, spiA* ve *csgA*) bahsi geçen tüm izolatlarda taranmıştır. Buna ilave olarak, ekstrakt, Mikawasima ve Virchow serovarlarının yüzme hareketlerini önemli ölçüde azalttığı belirlenmiştir. En yüksek azalma sırasıyla in vitro ve ıspanakta 0.88 Log CFU/mL ve 2.00 Log CFU/cm² olarak bulunmuştur.
Anahtar kelimeler: Antimikrobiyel etki, biyofilm, *Salmonella*, ısırgan otu, yüzme hareketliliği

INTRODUCTION

Due to health concerns, the demands to fresh produce have been increasing in the United States (US) and Europe (Faour-Klingbeil and Todd, 2018). Fresh food produces are generally consumed without any preprocessing processes. Therefore, if biofilms are present on the fresh produces, they might stay on the fresh product for a long time. Unless biofilms are removed, they might lead to foodborne diseases and shorten the shelf life of the products. In addition to this, contamination factors during growing, harvesting, handling, processing and transportation trigger biofilm formation of enteric pathogens such as *Salmonella enterica* spp. (Patel et al., 2013; Pyatkovskyy et al., 2017). Foodborne outbreaks associated with *Salmonella enterica* subp. *enterica* (*Salmonella*) serovars, due to contaminated fresh produce, including green leaves such as spinach, lettuce, alfalfa sprouts, and tomatoes, have been incrementally overspreading (Yaron and Römling, 2014). For instance, tomatoes contaminated with *Salmonella* serovars Typhimurium and Newport, packed salad contaminated with *Salmonella* serovars Enteritidis and Newport (Callejón et al., 2015) and salad items contaminated with *Salmonella* Virchow (Heaton and Jones, 2008) might be some examples. Moreover, *Salmonella* serovar Mikawasima, a rare serotype, has shown an unusual increase in outbreaks related to fresh waters, vegetables, meat and fish products (Synnott et al., 1993; Polo et al., 1999; EFSA and ECDC, 2013), and antibiotic resistance (Myšková et al., 2013) in the recent years. In addition to this, *Salmonella enterica* serovar Othmarschen, which is also an uncommon serovar, was isolated from eggs, seafood and squash (Kim et al., 2007).

Antibiotic resistance has become more critical problem, when these bacteria have biofilm forming ability, since biofilm enables bacteria to protect themselves from the environmental stresses such as chemical disinfectants and antibiotics. Hence, it becomes exceedingly difficult to remove bacteria. Biofilm formation might be expressed as clusters of bacterial cells encompassed by secreting extracellular polymeric substances (EPS) on abiotic and/or biotic surfaces in order to insulate themselves from environmental stresses, antibiotics, sanitizers, and the host (Steenackers et al., 2012). After aggregation and attachment of bacteria, they reveal quorum-sensing (QS) signals, which provide bacteria with communicating each other to regulate gene expression (Sperandio et al., 2003). Curli (amyloid fimbriae) are assigned in adherence to surfaces, biofilm forming, cell aggregation and host cell invasion (Römling et al., 1998). Flagellar motility enables *Salmonella* to swim in liquid media and on surfaces (Deditius et al., 2015), which might enhance the biofilm formation and pathogenicity.

Crude extracts of medicinal plants might function as alternatives to antibiotics, due to various secondary metabolites including tannins, alkaloids, and polyphenols (Gupta and Birdi, 2017). Plant extracts might change or inhibit the protein domains (Koehn and Carter, 2005). At this point, the detection of sufficient concentration of plant extract that can penetrate into the biofilm matrix to make the bacteria ineffective is crucial because EPS defends the microorganisms by constituting biological, physical and chemical protective barriers (Lu et al., 2019). Since each bacterium has different QS system and cell matrix, distinct types of plant extracts are applied in order to inhibit EPS production and QS signals. For example, the leaves of stinging nettle (*Urtica dioica*) include tannins taking parts in inhibition of cell envelope transport proteins and adhesiveness of bacteria, and terpenes participating in deterioration of membrane in bacteria (Gupta and Birdi, 2017). Water, hexane, chloroform, ethyl acetate and methanol extracts of stinging nettle have shown antimicrobial activities on human pathogenic bacteria such as *Pseudomonas aeruginosa*,

Staphylococcus aureus, *Salmonella* Typhi, *Klebsiella pneumoniae*, and *Enterococcus faecalis* (Gülçinet al., 2004; Daret al., 2013). Our aim in this study was to determine the eradication effects of aqueous extract of stinging nettle on biofilm forming of different *Salmonella* serovars *in vitro,* as well as on spinach leaves. Moreover, the effects of different concentrated water-soluble extracts of stinging nettle on swimming motility of each serovar were investigated *in vitro*. To sum up, the usage of alternative edible plant extracts has a huge potential on prevention of bacterial growth due to health concerns. Therefore, more studies should be conducted to determine the effective concentration of plant extracts that can also penetrate the biofilms of foodborne pathogens.

MATERIALS AND METHODS

Bacterial strains

Salmonella enterica serovar Virchow (MET S1-003), Newport (MET S1-166), Typhimurium (MET S1-185), Enteritidis (MET S1-217), Othmarschen (MET S1-227) and Mikawasima (MET S1-409), isolated from different sources and locations in our previous studies (Gunel et al., 2015; Acar et al., 2017), were used in this study (Table 1). Each serovar was grown in Brain Heart Infusion (BHI, CM1135 Oxoid) broth medium for 24 h at 37°C at 150 rpm to get the stationary phase cultures (Cui et al., 2016).

Preparation of the stinging nettle extract

Different concentrated crude extracts of lyophilized stinging nettle leaves, which are 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL, 20 mg/mL and 40 mg/mL, were preparedas described (Wolska et al., 2015). Lyophilized stinging nettle (*Urtica dioica* L.) leaves (obtained from Ankara, Turkey) in sterile ddH$_2$O at 90°C were shaken for10 min in a water bath, and then cooled to the room temperature (Wolska et al., 2015). The solution was vacuum-filtered by using a sterile mixed cellulose esters filter (0.45 µm, MF-Millipore membrane filter, Merck).

Detection of curli expression in *Salmonella* serovars

Salmonella serovars were grown in Tryptic Soy Broth (TSB, CM0129 Oxoid) for 18 h at 37°C. Overnight cultures of individual *Salmonella* serovars were streaked on tryptone agars containing congo red (40 µg/mL, C6767 Sigma-Aldrich) and coomassie brilliant blue (20 µg/mL, B0770 Sigma-Aldrich), and incubated at 22°C and 37°C for 48 h, respectively. Curli expression in strains was indicated by red colonies as a result of dye uptake (Patel et al., 2013).

Detection of swimming motility in *Salmonella* serovars

Bacterial strains in BHI (CM1135 Oxoid) broth medium were grown for 48 h at 37°C. Soft agar plates (1% tryptone, 0.5% NaCl and 0.3% agar) were inoculated with 2 µL of cell suspension, and incubated at 37°C for 24 h. Then, the diameters of bacterial growth were measured. Moreover, the effects of the extract on swimming motility were observed by using 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL and 20 mg/mL of stinging nettle aqueous extracts.

Biofilm formations and determining the antibiofilm effects of the stinging nettle extract *in vitro*

For each bacterial strain with six replicates, 190 µL of BHI supplemented with 10 µL of overnight cultures of individual *Salmonella* serovars with concentration of ca. 10^6 CFU/mL in BHI was dispensed into the wells of a 96-well flat-bottomed sterile polystyrene microtiter plate as described method with some modifications (Stepanovićet et al., 2004). Microtiter plates were incubated at 37°C for 48 h without shaking. Growth medium devoid of bacterial inoculum, and extract of stinging nettle served as negative control. After 48 h incubation in microplate, the medium from the well was discarded. The wells were washed five times with sterile phosphate-buffered saline (PBS, pH 7.2) to remove the planktonic cells, and were air-dried (Christensen et al., 1985). Subsequently, pre-formed biofilms in the wells were treated with 200 µL water-soluble extract of stinging nettle individually at different concentrations which are 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL and 20 mg/mL for 24 h at 37°C to determine biofilm eradication effect. The microtiter plates were washed with PBS to remove freely moving bacteria and stained with 200 µL of a 1% crystal

violet (CV) solution per well for 5 min at room temperature, followed by washing five times with sterile PBS and air drying for 45 min. 200 μL of 95% ethanol was added to each well for de-staining (Chen et al., 2013). The absorbance was determined by measuring at 600 nm (A_{600}) using spectrophotometer (SHIMADZU, Japan). *Salmonella* strains were divided into weak, moderate or strong producers, as described (Stepanović et al., 2004). The cut-off OD (ODc) was expressed as three standard deviations above the mean OD of the negative control. Classification was carried out as following: ODc< OD ≤ (2×ODc) for weak biofilm producer, (2×ODc) < OD ≤ (4×ODc) for moderate biofilm producer, and (4×ODc) < OD for strong biofilm producer (Stepanović et al., 2004).

Biofilm formations and determining the antibiofilm effects of the stinging nettle extract on spinach

Spinach leaves were aseptically cut into small rectangular pieces (4×4 cm). The vegetable pieces were submerged in each overnight culture of each bacterial strain with a concentration of ca. 10^6 CFU/mL. After overnight incubation at 25°C, the pieces were rinsed with sterile PBS to remove freely moving cells. They were transferred to sterile petri dishes and dried in the laminar flow cabinet for 15 min. For biofilm formation, contaminated spinach pieces were incubated at 25°C for 48 h in petri dishes (Cui et al., 2016). Individual spinach pieces, inoculated with each bacterial strain, were treated with 3 mL of the stinging nettle extract (40 mg/mL) for 5, 10, 30 and 60 min, respectively. Higher concentration of the extract was used on spinach leaves compared to *in vitro* since the eradication of biofilm formed on fresh produce is more troublesome, due to the surface area of it and rich medium of the fresh produce for the pathogens. After treatments with stinging nettle extract, 10 pieces of leaves were aseptically transferred into a stomacher bag and mixed with 100 mL PBS (Cui et al., 2016). Subsequently, the mixture was pureed by a homogenizer for 20 min. The numbers of viable bacteria on the leaves were determined by plate count method. Homogenized mixture was diluted in PBS to ranging dilution factor between 10^{-6} and 10^{-8}. Aliquots were spread out using a sterile bacterial spreader of each dilution onto the Plate Count Agar (CM0325, Thermo Fisher Scientific) plates. They were incubated at 37 °C for 48 h. The number of bacterial colonies were counted.

Screening of biofilm related genes in *Salmonella* isolates

Selected genes associated with biofilm formation (i.e. *rpoS, mlrA, ycf*R, *fimA, spiA,* and *csgA*) were screened in *Salmonella enterica* isolates (Table 2). PCR mix was prepared as 1 μL template, 1 μL each primer (20 μM), 25 μL 2X MyTaq Red Mix (Bioline), and 22 μL sterile ddH_2O for each gene according to MyTaq Red Mix protocol. PCR was performed with regard to the following conditions: initial denaturation at 95°C for 60 s, denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension 72°C for 10 s with 35 cycles (Ledeboeret al., 2006; Lu et al., 2011; Lu et al., 2012; Salazar et al., 2013; Uhlich et al., 2013; Han et al., 2018). 1.7% agarose gel was run at 100 V for 45 min, for confirmation.

Statistical Analysis

All experiments were carried out in six replicates. Statistical analysis was done by using Two Way Analysis of Variance (ANOVA). $P< 0.05$ was considered to be significant.

RESULTS AND DISCUSSION

In this study, we used different *Salmonella* isolates, representing most common serovars associated with foodborne outbreaks bound up with fresh produces, as well as serovars we collected from fresh produces previously in our laboratory (Gunel et al., 2015; Acar et al., 2017). *Salmonella* Virchow, collected from chicken meat, was the only multidrug-resistant (resistant to ampicillin, ceftiofur, nalidixic acid, streptomycin, sulfisoxazole, trimethoprim, and sulfamethoxazole-trimethoprim) serovar used in this study. Furthermore, *Salmonella* Newport, isolated from cattle, Typhimurium and Othmarschen, isolated from human clinical sources, showed antibiotic resistance to only sulfisoxazole, while *Salmonella* Enteritidis, collected from human clinical source, and Mikawasima, collected from iceberg were susceptible to all antimicrobials tested (Table 1).

Table 1. *Salmonella enterica* subsp. *enterica* isolates.

METU-IDs	Serovars	Phenotypic Antimicrobial Resistance Profile[a]	Source	Collected from City-Country
MET S1-003	Virchow	AmpEftNalStrSfTmpSxt	Chicken meat	Ankara-Turkey
MET S1-166	Newport	Sf	Cattle	Şanlıurfa-Turkey
MET S1-185	Typhimurium	Sf	Human	Şanlıurfa-Turkey
MET S1-217	Enteritidis	Susceptible	Human	Şanlıurfa-Turkey
MET S1-227	Othmarschen	Sf	Human	Şanlıurfa-Turkey
MET S1-409	Mikawasima	Susceptible	Iceberg	Ankara-Turkey

[a]Susceptible: Susceptible to antibiotics including amikacin, amoxicillin-clavulanic acid, ampicillin (Amp), cefoxitin, ceftiofur (Eft), ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, ertapenem, gentamicin, imipenem, kanamycin, nalidixic acid (Nal), streptomycin (Str), sulfisoxazole (Sf), sulfamethoxazole-trimethoprim (Sxt), tetracycline, trimethoprim (Tmp).

Curli expression

Flagella fulfilling adhesion, curli fimbriae, cellulose, lipopolysaccharides, and some outer membrane proteins are factors participating in adherence to surface. Curli proteins, such as amyloids, participate in colonization, durability, invasion and motility (Uhlich et al., 2013). Moreover, curli help to build attachment between cell and surface, and then the interactions among cell cultures (Steenackers et al., 2012). Furthermore, the previous studies revealed that curli expression depends on the strain and certain conditions (Barnhart and Chapman, 2006). Furthermore, curli are in need of initial phases of biofilm formation.

In our isolates, representing serovar Virchow was weak curli producer at 37 °C, while Newport, Typhimurium, Enteritidis, Othmarschen and Mikawasima serovars were strong curli producers at 37 °C. On the other hand, none of the isolates expressed curli production on congo red medium at 22 °C. Hence, the results indicated that the incubation temperature is a key point for curli expression of *Salmonella* isolates used in this study. In another research the greatest level of curli expression was observed by *S.* Seftenberg, followed by *S.* Enteritidis and *S.* Thompson, while *S.* Newport and *S.* Typhimurium developed lower intensity of colony staining with congo red at 28 °C (Patel et al., 2013). Yet, further studies are required to determine the certain inducements.

Effect of plant extracts on swimming motility

Different concentrated aqueous extracts of stinging nettle (i.e. 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL and 20 mg/mL) had various effects on swimming motility of *Salmonella* Virchow, Newport, Typhimurium, Enteritidis, Othmarschen, and Mikawasima serovars (Table 3). Swimming motility based on flagella enhances the adherence to surface and biofilm maturation. *Salmonella* isolates swim in liquid medium and surfaces by the help of flagellar motility triggering the biofilm formation and attachment to surface (Deditius et al., 2015). In general, when the bacteria get through to plant surfaces, motility and chemotaxis might help them to form biofilm in adherence areas. In plant-bacterial interactions, the bacteria respond to plant-produced signals, including spatial and nutritional signals (Mohan et al., 2018). Flagella and fimbriae play a crucial role in the mobility and attachment for settlement in the formative stages of biofilm development by bacteria on host plants (Mohan et al., 2018).

In our study, motility abilities of bacterial strains were determined according to the diameter (cm) of the halos grown on soft agar plates without stinging nettle extract as control. The highest ability for swimming motility was observed in *S.*

Virchow (8.5±0.01) followed by S. Typhimurium (8.0±0.02), *S.* Enteritidis (8.0±0.02), *S.* Othmarschen (8.0±0.01), *S.* Newport (7.5±0.01), and *S.* Mikawasima (6.0±0.02) on soft agar plates without extract (Table 3). Motility inhibition of extracts was ascertained as the percentage (%) decrease in diameter on soft agar plates. The most inhibitory effects on motility in percentages were found as 83.33% (*S.* Mikawasima, 1.0±0.22; 4 mg/mL) followed by 80.00% (*S.* Virchow, 1.7±0.13; 4 mg/mL), 55.00% (*S.* Othmarschen, 3.6±0.30; 6 mg/mL), 53.75% (*S.* Typhimurium, 3.7±0.24; 12 mg/mL), 40.00% (*S.* Newport, 4.5±0.58; 6 mg/mL), and 32.50% (*S.* Enteritidis, 5.4±0.62; 6 mg/mL) (Table 3). More interestingly, these highest inhibitory effects resulted from the usage of different concentrated aqueous extracts of stinging nettle, including 4 mg/mL, 6 mg/mL, and 12 mg/mL. While 4 mg/mL concentrated stinging nettle extract inhibited the mobility of *S.* Virchow and Mikawasima serovars in considerable level, 6 mg/mL extract had inhibitory effect on *S.* Othmarschen, Newport, and Enteritidis serovars. Additionally, in case of *S.* Typhimurium, 12 mg/mL extract was determined as the most inhibitory concentration for the swimming motility. Although the concentrations of the extract were different, the highest similar reductions were ascertained in swimming motilities of Typhimurium and Othmarschen serovars (Table 3).

Table 2. Selected *Salmonella* biofilm associated genes.

Gene	Primer Sequence (5'→ 3')[a]	Function	Reference
rpoS	F: TATCGCCTGGATTACTGGCAAC R: TAGGACGCTGACGTGTCTTATC	Stationary-phase sigma factor	Uhlich et al., 2013
mlrA	F: ACATACCCGCAAACCACACTTC R: AGCTATGCGCATAATGCACTCC	Regulator of curli and extracellular matrix formation	Uhlich et al., 2013
*ycf*R	F: ACGCCAGAAGGTCAACAGAA R: GGGCCGGTAACAGAGGTAA	Putative membrane protein included in biofilm	Han et al., 2018; Salazar et al., 2013
fimA	F: TTGCGAGTCTGATGTTTGTCG R: CACGCTCACCGGAGTAGGAT	Fimbrial unit	Ledeboer et al., 2006
spiA	F: GAGATATGCCATTATTTACTACTGAGG R: TGACTTCACCCTTTATGCCAGAC	Outer membrane protein involved in biofilm	Lu et al., 2012
csgA	F: TATTATCCGCACCCTGGCCTAC R: GCAATAGTTCCGGCCCG	Curli subunit	Lu et al., 2011

[a]F: Forward Primer, R: Reverse Primer.

To the best of our knowledge, this is the first study determining the effects of different concentrated aqueous extracts of stinging nettle (*U. dioica*) on swimming motilities of *Salmonella* serovars. In another research, 5 mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL aqueous extract of stinging nettle reduced the motility of *Escherichia coli* rods as ~22%, ~6%, ~14%, and ~34%, respectively, while 0.125 mg/mL increased the motility ~32% (Wojnicz et al., 2012). Besides that, motilities of *S.* Newport, *S.* Typhimurium, and *S.* Enteritidis increased in agar supplemented with apitoxin (512 µg/mL) compared to control without apitoxin in another study (Arteaga et al., 2019).

Our results showed that some concentrations reduced the bacterial motility, while others increased it as well. It is difficult to explain this phenomenon, because there are no reports defining these kinds of points. However, another study demonstrated that bacteria growing at pH

6.0 and 7.0 were more motile than those grown at pH 5.8-10.0 (Hattermann and Ries, 1989). Further studies are required in order to understand the effects of different concentrated extracts on swimming motiliy. It might be concluded that particular concentration is required to inhibit considerably the swimming motility of each serovar. In addition to this, aqueous extract of stinging nettle might play a crucial role in limiting the motilities of *Salmonella* serovars during the formative stages of biofilm development on host plants.

Table 3. Swimming mobility capacities of biofilm forming *Salmonella* isolates in presence of the aqueous extract of stinging nettle in different concentrations.

Serovars	Control[a] (cm)	Diameter (cm) on plates, including extract of stinging nettle[b]							Decrease in diameter[c] (%)
		2 mg/mL	4 mg/mL	6 mg/mL	8 mg/mL	12 mg/mL	16 mg/mL	20 mg/mL	
Virchow	8.5± 0.01	8.5± 0.01	**1.7± 0.13**	5.0± 0.28	8.5± 0.03	6.4± 0.14	7.0± 0.20	7.0± 0.01	80.00
Newport	7.5± 0.01	7.5± 0.01	4.8± 0.25	**4.5± 0.58**	5.1± 0.26	5.6± 0.23	5.5± 0.16	5.5± 0.23	40.00
Typhimurium	8.0± 0.02	8.0± 0.02	5.5± 0.38	5.1± 0.41	6.8± 0.32	**3.7± 0.24**	5.9± 0.46	6.5± 0.38	53.75
Enteritidis	8.0± 0.02	8.0± 0.02	8.0± 0.10	**5.4± 0.62**	7.5± 0.09	7.5± 0.02	7.5± 0.10	7.5± 0.12	32.50
Othmarschen	8.0± 0.01	8.0± 0.01	4.5± 0.32	**3.6± 0.30**	4.5± 0.15	7.5± 0.03	7.5± 0.05	7.5± 0.02	55.00
Mikawasima	6.0± 0.02	6.0± 0.02	**1.0± 0.22**	5.0± 0.35	6.5± 0.07	5.5± 0.04	6.5± 0.04	8.5± 0.03	83.33

[a]Agar medium of control groups includes 1% tryptone, 0.5% NaCI and 0.3% agar without stinging nettle extract.
[b] Agar media consists of 1% tryptone, 0.5% NaCI, 0.3% agar, and different concentrated extracts of stinging nettle, which are 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL and 20 mg/mL.
[c] Diameters were measured according to the most inhibitory concentration specified as bold number narrow for each serovar. Calculation was done as specified: [100 - (Diameter in the most inhibitory concentration×100/ Diameter in the control)]. Diameters are the average values of the six replicates. *P*-value< 0.05 for stinging nettle concentrations used to inhibit swimming mobility. There is a significant difference between stinging nettle concentrations.

Effect of plant extracts on pre-formed biofilms *in vitro*

Optical density at 600 nm (OD_{600}) for each *Salmonella* serovar was found as 2.2802±0.30 (Mikawasima), 2.2321±0.26 (Othmarschen), 2.0389±0.24 (Enteritidis), 0.8502±0.15 (Typhimurium), 0.7985±0.10 (Virchow), and 0.6201±0.01 (Newport) *in vitro* as control groups untreated with stinging nettle extract, respectively (Table 4). Mikawasima, Othmarschen and Enteritidis serovars were determined as strong biofilm producers, while Typhimurium, Virchow and Newport serovars were ascertained as moderate biofilm producers *in vitro*, as described classification (Stepanovićet al., 2004). Although Mikawasima is a rare serovar, in this study Mikawasima was found out as the strongest biofilm producer. In another study, biofilm development capacity of *S.* Mikawasima isolated from laying hens was determined as the highest compared to Enteritidis, Typhimurium, and Virchow serovars as well (Marin et al., 2009). Another rare human pathogen, which is *S.* Othmarschen, was also found as one of the strongest biofilm producers in this study. On the other hand, *S.* Newport, which was characterized as a strong biofilm producer in a broad array of environmental conditions in another study (Lianou and Koutsoumanis, 2012), was typified as a moderate biofilm producer in our study. Different biofilm forming abilities in *Salmonella* serovars are strongly considered to derive from strain-dependence (Lianou and Koutsoumanis, 2012).

Additionally, eradication effects of water-soluble stinging nettle extracts including 2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL, and 20 mg/mL on pre-formed biofilm formations of *Salmonella* serovars were specified (Table 4). The findings indicated that for each serovar these extracts had different eradication effects on pre-formed biofilm. For instance, the highest Log CFU/mL reduction (0.55±0.16) on pre-formed *S.* Mikawasima biofilm was observed using 2 mg/mL aqueous extract of stinging nettle. In *S.* Othmarschen 0.88±0.20 Log CFU/mL reduction was noticed applying 20 mg/mL extract. Surprisingly, higher concentration (20 mg/mL) partially eradicated biofilm formation of Othmarschen serovar, while lower concentration (2 mg/mL) resulted in the highest eradication effect on biofilm of Mikawasima serovar. It is difficult to explain the reason of this finding. Yet, the complex interactions governing biofilms might be distinctly affected by the components of stinging nettle extract. The greatest reduction in pre-formed biofilm of *S.* Enteritidis *in vitro* was obtained as 0.68±0.20 Log CFU/mL within 4 mg/mL extract. Among the strong biofilm producers including Mikawasima, Othmarschen and Enteritidis serovars, water-soluble stinging nettle extract had the greatest eradication effect on pre-formed biofilm of Othmarschen. On the contrary, in moderate biofilm producers, which are Typhimurium, Virchow and Newport serovars, 0.08±0.02 Log CFU/mL (2 mg/mL), 0.28±0.12 Log CFU/mL (4 mg/mL), and 0.30±0.15 Log CFU/mL (4 mg/mL) were found as the greatest reductions, respectively (Table 4). Findings showed that aqueous stinging nettle extract did not have any pivotal effect on eliminating pre-formed biofilm of Typhimurium serovar, while same concentrated extract (4 mg/mL) demonstrated similar eradication effect on biofilm formations of Virchow and Newport serovars. More interestingly, no reduction on biofilms of Typhimurium and Newport was determined using 8 mg/mL extract although lower and higher concentrations than 8 mg/mL indicated some reductions (Table 4).

Table 4. The Log CFU/mL reductions of *Salmonella* isolates *in vitro* with the aqueous extract of stinging nettle in different concentrations.

Isolates METU-IDs	Serovars	Control, OD_{600}	Log CFU/mL Reduction[a]						
			2 mg/mL	4 mg/mL	6 mg/mL	8 mg/mL	12 mg/mL	16 mg/mL	20 mg/mL
MET S1-003	Virchow	0.7985± 0.10	0.17± 0.05	**0.28± 0.12**	0.24± 0.17	0.22± 0.14	0.23± 0.11	0.26± 0.10	0.25± 0.10
MET S1-166	Newport	0.6201± 0.01	0.27± 0.10	**0.30± 0.15**	0.28± 0.16	No reduction	0.13± 0.05	0.25± 0.10	0.27± 0.11
MET S1-185	Typhimurium	0.8502± 0.15	**0.08± 0.02**	0.01± 0.01	0.02± 0.01	No reduction	0.02± 0.01	0.05± 0.02	0.01± 0.01
MET S1-217	Enteritidis	2.0389± 0.24	0.66± 0.13	**0.68± 0.20**	0.35± 0.09	0.25± 0.18	0.49± 0.18	0.48± 0.13	0.54± 0.16
MET S1-227	Othmarschen	2.2321± 0.26	0.57± 0.11	0.60± 0.14	0.70± 0.16	0.85± 0.20	0.83± 0.21	0.14± 0.04	**0.88± 0.20**
MET S1-409	Mikawasima	2.2802± 0.30	**0.55± 0.16**	0.23± 0.11	0.26± 0.12	0.32± 0.12	0.35± 0.10	0.47± 0.10	0.48± 0.15

[a]Optical densities of each control group were calculated by taking the average values of the six replicates. Bold values were representing statistically significant values between Log CFU/mL reductions resulting from the usage of different concentrated extracts of stinging nettle with P-value < 0.05.

It might be concluded that for each serovar there is a certain threshold for the concentration of stinging nettle extract in order to eradicate the pre-formed biofilms. To understand deeply the reason of these, more studies related to biofilm matrix are required. In another study, the use of glutaraldehyde, formaldehyde, and peroxygen at concentration of 1.0% in field conditions was determined as inadequate for *Salmonella* elimination irrespective of the serotype, the biofilm development capacity, and the disinfectant contact time (Marin et al., 2009). In

addition to this, aqueous extract of stinging nettle used in this study demonstrated better elimination of biofilms compared to most common disinfectants including glutaraldehyde, formaldehyde, and peroxygen. Apitoxin destroyed the pre-formed biofilms of *S*. Enteritidis, Newport, and one of Typhimurium strains on polystyrene surface as 0.16 Log, 0.28 Log, and 0.40 Log reduction, respectively (Arteaga et al., 2019). In comparison with this result, our study showed higher eradication effect on pre-formed biofilms of Enteritidis and Newport. Moreover, carvacrol and thymol, which are the main components of oregano and thyme oils, reduced *S*. Typhimurium biofilms about 5 Log, and *S*. Enteritidis biofilms about 4 Log on polypropylene surface (Amaralet al., 2015). Although carvacrol and thymol indicated much greater destructive effect on biofilm formations compared to stinging nettle, the roles of biofilm formation surfaces on eliminating effect should also be taken into consideration. Nevertheless, a little or nothing is known related to eradication effects of plant extracts on pre-formed biofilms of *S*. Mikawasima, Othmarschen, Newport and Virchow serovars. Therefore, our study might contribute important findings to literature about disruptive effects of aqueous extract of stinging nettle on pre-formed biofilms of *Salmonella* serovars *in vitro*.

Effect of plant extract on pre-formed biofilms on spinach leaves

Surface structures and genotypic features of the plant, the regional physicochemical properties, the ecological conditions, such as temperature, wind, soil and rainfall, and the processes from field to fork have crucial impacts on the biofilm forming ability of microbial communities on plants (Carter and Brandl, 2015). Moreover, biofilms on fresh produce enable bacteria to endure against environmental stress conditions, exchange genetic elements, such as antibiotic resistance genes among bacteria, and interoperate in metabolic and physical way. Thus, 40 mg/mL, a higher concentration compared to the ones used for *in vitro*, was applied to the biofilms *Salmonella* serovars on spinach leaves, since eradication of biofilms on fresh produce has been harder than of *in vitro*. Furthermore, exposure time also plays a crucial role in obstructing the biofilm formation on fresh products. In other words, efficient exposure time is required to impair the biofilm matrix of bacteria, and diffuse the bioactive compounds of the extracts and/or essential oils into the bacterial cells.

Salmonella colonies on samples without treatments were counted as controls. Initial microbial loads of *Salmonella* serovars were recovered from spinach leaf surface as 8.30±2.00 Log CFU/cm^2 (*S*. Mikawasima), 8.30±1.00 Log CFU/cm^2 (*S*. Othmarschen), 8.00±2.00 Log CFU/cm^2 (*S*. Enteritidis), 7.95±2.00 Log CFU/cm^2 (*S*. Virchow), 7.78±1.00 Log CFU/cm^2 (*S*. Newport), and 7.30±1.00 Log CFU/cm^2 (*S*. Typhimurium), respectively (Table 5). Biofilm forming ability on spinach leaf surface was found similar between *Salmonella* serovars ($P > 0.05$) without exposure to water-soluble stinging nettle extract. In another study, *Salmonella* Tennessee and *S*. Thompson produced stronger biofilms *in vitro* compared to *S*. Newport, *S*. Negev, and *S*. Braenderup attached more strongly to lettuce than other serovars (Patel and Sharma, 2010). Furthermore, *S*. Typhimurium growth was inhibited when cultured in rocket extract and when grew directly to rocket leaves (Doulgeraki et al., 2016). Another research indicated that *Salmonella* persistence on spinach leaves is affected by the source of contamination and the biofilm forming ability of the strain (Patel et al., 2013). Although *Salmonella* serovars used in this study were isolated from different sources including chicken meat, cattle, human, and iceberg, and *S*. Typhimurium, Virchow and Newport serovars ascertained as moderate biofilm producers *in vitro,* all serovars demonstrated similar biofilm forming ability on spinach leaf. Moreover, *S*. Virchow showed a little higher biofilm forming capability on spinach leaf compared to that of representing Newport and Typhimurium serovars despite being weak producer (*S*. Virchow) in curli expression. The reason of that might be connected to strain and characteristics of the biofilm formation. In addition to this, greater biofilm forming ability of *S*. Mikawasima and Othmarschen, which are rare serovars compared

to *S*. Enteritidis, Typhimurium, Virchow and Newport, on spinach might pose a serious threat to food safety. In other words, if leafy green vegetables consumed as raw salad are contaminated with pathogenic *Salmonella*, the eradication of them from fresh produce gains prominence.

Different exposure time, including 5 min, 10 min, 30 min, and 1 h, of 40 mg/mL aqueous extract of stinging nettle were applied to pre-formed biofilms of *Salmonella* serovars. The greatest reductions were recovered for *S*. Newport, Enteritidis, Othmarschen, Mikawasima, Typhimurium, and Virchow serovars as 2.00±0.21 Log CFU/cm² (1 h exposure of extract), 1.70±0.23 Log CFU/cm² (1 h exposure of extract), 1.70±0.26 Log CFU/cm² (1 h exposure of extract), 1.70±0.18 Log CFU/cm² (1 h exposure of extract), 1.40±0.21 Log CFU/cm² (30 min exposure of extract), and 0.88±0.20 Log CFU/cm² (5 min exposure time), respectively (Table 5). The results demonstrated that serovar-dependence is also important information as well as time-dependence for the eradication efficiency of water-soluble stinging nettle extract on pre-formed biofilms of *Salmonella* serovars ($P < 0.05$). For instance, 5 min treatment with 40 mg/mL stinging nettle extract exhibited the greatest disruption performance on pre-formed biofilm of *S*. Virchow, while no reduction was observed on pre-formed biofilm of *S*. Mikawasima which is an emerging serovar causing outbreaks recently. Besides, 5 min treatment was found as the second highest effective exposure time for the pre-formed biofilm of *S*. Newport. In contrast, 30 min treatment showed a considerable eradication effect on *S*. Typhimurium, which is a common serovar associated with numerous foodborne outbreaks. Additionally, greater considerable reductions were determined within 1 h exposure of the extract on biofilms of *S*. Newport, Enteritidis, Othmarschen, and Mikawasima serovars. Even though it is hard to clarify the interaction between serovar-dependence and time-dependence to eliminate pre-formed biofilm within the extract, importance of exposure time of the stinging nettle extract on biofilm formations of each *Salmonella* serovar were enlightened to observe antibiofilm effects on spinach leaves.

Table 5. The Log CFU/cm² reductions of *Salmonella* isolates on spinach leaf at different exposure times with 40 mg/mL stinging nettle extract.

Isolates METU-IDs	Serovars	Initial Microbial Load, (Log CFU/cm²) [a]	Log CFU/cm² Reduction[b]			
			5 min	10 min	30 min	1 h
MET S1-003	Virchow	7.95±2.00	**0.88±0.20**	No reduction	0.21±0.05	0.80±0.11
MET S1-166	Newport	7.78±1.00	1.52±0.12	0.77±0.14	0.68±0.15	**2.00±0.21**
MET S1-185	Typhimurium	7.30±1.00	1.00±0.15	0.82±0.23	**1.40±0.21**	0.70±0.20
MET S1-217	Enteritidis	8.00±2.00	0.49±0.13	0.06±0.01	1.40±0.10	**1.70±0.23**
MET S1-227	Othmarschen	8.30±1.00	0.15±0.09	0.14±0.03	1.10±0.20	**1.70±0.26**
MET S1-409	Mikawasima	8.30±2.00	No reduction	0.20±0.05	1.00±0.10	**1.70±0.18**

[a] CFU: Colony Forming Unit. Colony forming units were given as the average values of the six replicates.
[b] Bold values were representing significant difference between exposure times with *P*-value < 0.05.

Studies related to the eradication of pre-formed biofilms of *Salmonella* on biotic surfaces such as plants within plant extracts are limited and/or not found in the literature. A study detected 2.26 Log CFU/cm² reductions in lettuce samples inoculated with *E. coli* O157:H7 after 4 mg/mL clove oil treatment for 30 min (Cui et al., 2016). Rather than biotic surfaces, there are more studies about elimination of biofilm formation by *Salmonella enterica* on different food-contact surfacesusing essential oils, plant extracts and/or chemical disinfectants in the literature. For example, the levels of *Salmonella* spp. biofilm cells were reduced by 3.3 Log CFU/cm² when treated

with thyme essential oil and by 3.1 Log CFU/cm^2 when treated with tea tree essential oil at 0.1% v/v within 2 h-exposure on stainless steel surface (Sadekuzzaman et al., 2018). However, the reductions were lesser on rubber surface than those found on the stainless steel surface (Sadekuzzaman et al., 2018). More interestingly, 1, 15, or 60 min exposure of chemical solutions which are glutaraldehyde, formaldehyde, and hydrogen peroxide at 1.0% concentration were inadequate for *Salmonella* elimination on abiotic surfaces (Marin et al., 2009). Our findings might contribute to the literature about the elimination of pre-formed biofilms of *Salmonella* on raw vegetables such as spinach using water-soluble stinging nettle extract.

Biofilm related genes

In molecular level, biofilm associated genes (i.e.*rpoS, mlrA, ycf*R*, fimA, spiA and csgA*) were screened in all *Salmonella* isolates including Virchow, Newport, Typhimurium, Enteritidis, Othmarschen and Mikawasima serovars in this study. *rpoS* is an important control unit in expression of many genes related to stress response, regulation and metabolism (Chen et al., 1996). RpoS, stationary-phase sigma factor, is also assigned in curli gene expression (Arnqvist et al., 1994) and cellulose production (Wang et al., 2010). Moreover, *rpoS* plays a crucial role in adhering to the plants for *Salmonella enterica* (Wang et al., 2010). RpoS regulates the curli gene expression by actuating MlrA expression as well (Brown et al., 2001). Thus, MlrA takes part in the generation of RpoS-based curli, and extracellular matrix formation of *Salmonella* isolates. YcfR promotes the surface attachment in *Salmonella* and triggers biofilm formation (Salazar et al., 2013). In another research, it was ascertained that the deletion of *ycf*R in *Salmonella enterica* serovar Typhimurium and Saintpaul decreased the attachment of them to spinach leaves after chlorine treatment (Salazar et al., 2013). *fimA,* encoding the major fimbrial protein subunit (Cohen et al., 1996), takes charge in adherence, pathogenesis and colonization during the biofilm formation (Zeiner et al., 2012). *spiA* secreting type III outer membrane protein is assigned to virulence factors in biofilm formations, and curli expression (Dong et al., 2011). CsgA which is the major structural subunit is expressed by *csgBAC* operon required for curli secretion in *Salmonella* (Römling et al., 1998). In this study, among *Salmonella* Virchow, Newport, Typhimurium, Enteritidis, Othmarschen and Mikawasima serovars, *rpoS, mlrA, ycf*R*, fimA, spiA,* and *csgA* genes were detected. Our findings as regards selected biofilm formation related genes (Table 2) were compatible by taking into consideration the biofilm forming abilities of *Salmonella* serovars *in vitro* and spinach, and curli expression except for *Salmonella* Virchow, due to being weak in curli production. However, further investigation should be conducted to determine the expression levels of each gene in biofilm production *in vitro* as well as on spinach leaves.

CONCLUSION

All in all, this study indicated various eradication impacts of the different concentrated aqueous extracts of stinging nettle (*Urtica dioica*) on biofilm forming foodborne pathogen *Salmonella enterica* isolates. Cellular properties of the strains, bioactive constituents of the stinging nettle, and QS molecules and extracellular polysaccharides of biofilm forming *Salmonella* might be adduced for the reasons of distinct destructive effects. Moreover, the results showed that the significance of the exposure time of the extract in order to disrupt the biofilm structure on spinach. In conclusion, biofilm forming abilities of *Salmonella* isolates, representing Virchow, Newport, Typhimurium, Enteritidis, Othmarschen and Mikawasima serovars, were affected by different concentrations of aqueous extract of stinging nettle (*Urtica dioica*) in this research. By taking into consideration the natural, antimicrobial, nourishing features of stinging nettle, it might contribute to solution for biofilm spreading on fresh produce, which is a common problem of the world.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Aylin Cesur performed research. Aylin Cesur and Yeşim Soyer analyzed data, designed research, and wrote the paper.

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