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Research article

BIOFILM FORMING ABILITY OF *SALMONELLA* ENTERITIDIS *IN VITRO*

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Salmonella enterica serotype Enteritidis is an important alimentary pathogen that recently gained special attention due to the ability of a large number of strains to form biofilms. Qualitative testing of biofilm forming ability was performed by observing the morphotype of the colonies on Congo Red agar and by conducting the pellicle test, while quantitative testing was carried out by Cristal violet assay on microtiter plates. A total of 14 isolates of S.Enteritidis were tested for biofilm forming ability, while Salmonella Enteritidis ATTC 13076 was used as the reference strain. Based on the morphotype of colonies cultivated on Congo Red agar at 25°C incubation temperature, among tested isolates three morphotypes were detected – red, dry and rough (rdar), brown, dry and rough (bdar) and smooth and white (saw). Half of the tested isolates demonstrated rdar morphotype. All isolates that showed a specific morphotype at this incubation temperature also formed the corresponding type of pellicle at the air-liquid interface. Additionally, comparing OD (optical density) values obtained by crystal violet test between groups of isolates that represent one of the three detected morphotypes (rdar, bdar and saw), statistically significant differences were detected. Based on OD values obtained by crystal violet test at both applied incubation temperatures, isolates were classified into three categories, regarding their ability to form biofilms: strong, moderate and weak biofilm producers. By comparing the amounts of the biofilms formed after 48h at 25°C and 37°C, statistically significant differences were noted ($P<0.05$). In this research we presented micrographs and a reconstruction of three-dimensional projections of biofilm developing phases of rdar morphotype isolates, which were obtained using confocal laser scanning microscopy.

Key words: *Salmonella* Enteritidis, biofilm, curli fimbriae, cellulose

INTRODUCTION

In response to environmental conditions, bacteria have developed a variety of strategies to adapt and survive. The formation of multicellular communities known as biofilms

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is one such strategy, which is generally associated with the persistence and survival in different environmental conditions.

Biofilms formed by bacteria on different surfaces present a long-term source of contamination of foodstuffs in the food industry, not only with bacteria causing their spoilage but also with food-borne species such as *Salmonella* spp., *Campylobacter* spp., *Escherichia coli* and *Listeria monocytogenes* [1-6]. It is known that some food-borne pathogens in food production plants may exist throughout several months, even years. These strains are known as „house strains", and the assumption is that the existence of such strains is enabled due to their ability to form biofilms [4].

The food-borne pathogen *Salmonella enterica* has been associated with numerous cases of foodborne infections worldwide. According to scientific reports of EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), *S*. Enteritidis was the most frequently reported serotype (41.3 %) implicated in outbreaks of human salmonellosis in EU [7]. Also in Serbia, *Salmonella* Enteritidis is considered to be the most important foodborne pathogen followed by *Salmonella* Typhimurium [8,9]. The frequent involvement of serotype Enteritidis in outbreaks of human salmonellosis may be a consequence of the propensity of some strains to form biofilms, but this still remains to be firmly proved. Several studies confirmed the ability of adherence and biofilm formation of foodborne pathogen *S*. Enteritidis on different types of materials that are usually used in the food industry: stainless steel, glass, polystyrene, polyethylene, polypropylene, and granite [10-17].

Subsequent development of biofilms often occurs along with the production of extracellular polymers. *Salmonella* spp. produces an extracellular matrix with curli and cellulose as the major components. Curli are amyloid fibers which are involved, among other things, in the adhesion to surface, cell aggregation, environmental persistence and biofilm formation [18]. As a second matrix-component *Salmonella enterica* strains produce cellulose. Cellulose production confers bacterial cell–cell interactions, adhesion to abiotic surfaces (biofilm formation) and different types of resistance [19-22].

Depending on the expression of these extracellular components of *Salmonella* and other *Enerobacteriaceae,* they can display four morphotypes on indicator agar plates supplemented with Congo red and Coomassie brilliant blue. The curli and cellulose positive phenotype can be identified as violet to red, dry and rough (rdar). Cells lacking synthesis of cellulose will appear brown, dry and rough (bdar). However, if there is cellulose but no curli fimbriae, then the colony will appear pink, dry and rough (pdar). Phenotype isolates that do not express any of these two components form colonies which appear smooth and white (saw) [20,23]. Previous studies have indicated that *Salmonella* matrix components, such as curli fimbriae and cellulose i.e. rdar morphotype, play an important role in the survival of *Salmonella* in the environment [24-26].

Zogaj *et al.,* [21] demonstrated that natural *Salmonella* serotype Enteritidis isolates showed the rdar morphotype previously described only in *Salmonella* serovar

Typhimurium. Further occurrence of rdar morphotypes among different isolates of *S*. Enteritidis has been demonstrated in previous reports [22,27,28].

However, to the best of our knowledge, besides the mentioned reports there are no other available reports in the context of biofilm production of *S*. Enteritidis regarding to its morphotype. Also, after review of the available literature, our findings indicate that this is the first study in which *Salmonella* Enteritidis ATCC 13076 is used as a reference strain.

MATERIALS AND METHODS

Microorganisms

A total of 15 *S*. *enterica* strains of serovar Enteritidis were used in this study. Tests were performed with 14 isolates of *Salmonella* Enteritidis (SE2-SE15) obtained from the Veterinary Institute in Kraljevo, Serbia and from the Institute of Veterinary Medicine in Novi Sad, Serbia. *Salmonella* Enteritidis ATCC 13076 (lyophilized cultures of microorganisms, American Type Culture Collection, Kwik-stickTM set, MicroBioLogics) was used as the reference strain. Serological typing and verification of *Salmonella* isolates were carried out in the National Reference Laboratory for *Salmonella, Shigella, Vibrio cholera* and *Yersinia enterocolitica*, Institute of Public Health of Serbia „Dr Milan Jovanovic Batut", Belgrade, Serbia.

Preparation of bacterial suspension

All strains of *S*. Enteritidis were cultured on Tryptone Soya agar (TSA, LabM) and incubated at 37°C for 24h. Three to four isolated colonies grown on TSA were inoculated in 5 mL of Tryptone Soya broth (TSB, Oxoid) and incubated at 37°C for 18h. After incubation, suspensions were homogenized and diluted to 1:40 in fresh TSB. The density of the suspensions used for biofilm formation was adjusted to 0.5 McFarland standard turbidity (~1-2 x 10^8 CFU/mL) using densitometer DEN-1 (Biosan, Riga, Latvia) and standard plate count method from a series of ten-fold dilutions.

Biofilm production assays

Colony morphology

Colony morphology was analyzed on Luria-Bertani (LB) agar without salt and supplemented with Congo Red (40 μg/mL) and Coomassie brilliant blue (20 μg/mL), using Congo Red assay according to Malcova *et al.,* [29]. Colony morphology was determined after 96h incubation at 25°C and 37°C. The experiment was repeated on three separate occasions.

Biofilm phenotype at the air-liquid interface

To study biofilm phenotype at the air-liquid interface, i.e. ability to form pellicles at the air-liquid interface, we used the method by Vestby *et al.,* [30], with some modifications. More specifically, 1.8 mL of TSB was inoculated with 0.2 mL of a suspension of each isolate, and incubated for 96 hours at 25°C and 37°C.

For the purposes of comparative analysis of test results, we introduced a classification based on the intensity of pellicle production at the air-liquid interface according to the following scheme: 1) formation of strong and thick pellicle (+++) - strong biofilm producer, 2) formation of thin pellicle (++) - moderate biofilm producer, 3) formation of very thin pellicle (+) - weak biofilm producer, 4) complete absence of pellicle (-) - lack of ability to produce biofilm. Test was repeated three times for each isolate tested.

Crystal violet assay (CV)

The biofilm formation assay applied in the present study was based on previously described procedures, with some modifications [31-33]. More specifically, three wells of a sterile flat-bottomed 96-well polystyrene microtitre plates (Greiner Bio-One) were filled with 180 μL of TSB. Afterwards, aliquots of 20 μL of bacterial suspension (with the characteristics described in section - preparation of bacterial suspension) of each one of the *S*. Enteritidis strains were added into each well. Negative control wells contained only 200 μL of TSB per well. The plates were sealed and incubated for 48h at 25°C and 37°C. After incubation the content of the wells was discarded and, in order for non-adherent (or reversibly attached) bacterial cells to be removed, the wells were washed three times with 250 μL of sterile water. The remaining attached bacteria were fixed by drying in an inverted position at room temperature for 15 min. Subsequently each well was stained with 250 μL of 0.5% crystal violet for 10 min. Excess stain was rinsed off by filling the wells with sterile distilled water and emptying them by inversion of the plates for a total of six times. The microtiter plates, after vigorous tapping on absorbent paper, were air dried for 1h. The crystal violet bound to the formed biofilm mass was solubilized in 250 μL of decoloring solution for 15 min (ethanol/acetone, 80:20%) which was added to each well. The optical density of the wells was measured at 595 nm (OD_{595} nm) using an automated microtiter reader (ChemWel, Awareness Technology). The average value of the optical density (OD) measurements of the negative control wells in each microtiter plate was subtracted from the OD_{595} of each test well in the same plate and this difference referred to $\Delta OD_{595.}$ ΔOD_{595} was used for the characterization of the biofilm-forming ability of the tested isolates. According to these values, isolates were classified into 4 categories according to Stepanović *et al.*[31]. Whereby a limit value ODc (cut-off) was defined on the basis of OD values obtained for each test isolate and negative control (only broth) using an interactive dot diagram (MedCalc, StatSoft). Tests were performed in triplicate for each isolate and repeated twice on different days. Results were presented as the median value of the six replicates.

Biofilm formation on glass

Glass coupons (1 x 1 x 0.2 cm) were used for the examination of biofilms using confocal laser scanning microscopy (CLSM). The coupons were previously washed in detergent solution and rinsed with sterile distilled water. Afterwards, they were sterilized in an autoclave (Tutnauer, ELV 3870) for 15 minutes at a temperature of 121°C. Suspension preparation was carried out according to the protocols described in the previous sections. Each coupon was appointed separately into the recesses of a sterile polystyrene 12-well plate (Greiner Bio-One). Suspension of the test bacterial isolate was inoculated in an amount of 100 mL to the surface of each coupon. The adhesion of bacteria was provided during the 3 h incubation at 25°C. Afterwards, suspension was removed by aspiration. Coupons were washed with sterile physiological saline solution, and then submerged in 2 mL of TSB. Coupons were incubated for 24 and 48 h at 25°C. Immediately before the beginning of the coupons preparation for visualization of biofilms using CLSM, coupons were removed from the well and washed by gently pipetting with 3 mL of sterile saline to remove the medium and non-adhered cells.

Confocal laser scanning microscopy (CLSM)

For the purpose of visualization of biofilms using CLSM each coupon was appointed in 2 mL of phosphate buffered saline in a separate well. Prior to examination by CLSM, biofilms were fluorescently stained with 32 mM fluorescein diacetate (FDA, Sigma) and 7.5 mM propidium iodide (PI, Sigma). Viable bacteria with intact cell membranes were stained in green, whereas dead bacteria with damaged membranes were stained in red. Visualization of adherent cells was performed after incubation for 30 minutes at room temperature in the dark, using a confocal laser scanning microscope (LSM 510, Carl Zeiss GmbH, Jena, Germany) equipped with Ar Multi-line (457, 478, 488, and 514 nm) and HeNe (543 nm) lasers. Archoplan 40x/0.8W objective in water immersion was used for image acquisition. Following acquisition, images were processed using the Zeiss LSM Image Browser 4.2. Three-dimensional projections of biofilm structure were reconstructed using the software package BioImage XD [34].

Statistical analysis

Statistical analysis was performed with Statistica 12 (StatSoft Inc., Tulsa, Oklahoma) software. Because of the size of the sample ($n<30$), the data from the Crystal violet assay was compared using the nonparametric Mann-Whitney test. Comparison of the obtained results in the used assays was tested by Kruskal-Wallis test that was followed by multiple comparison of mean rank test. Results were considered to be statistically significant at $p<0.05$.

RESULTS

Biofilm production

Colony morphology

After 96h incubation at 25°C, three morphotypes (rdar, bdar and saw) were detected on LB agar, without NaCl supplemented, with Congo Red and Coomassie Brilliant Blue (Fig. 1.). Previously described rdar morphotype was expressed by seven isolates (SE5, SE7, SE11-SE15), while five isolates (SE2, SE4, SE6, SE9 and SE10) and the referent strain (SE1) expressed bdar morphotype. Morphotype saw was observed in two isolates (SE3 and SE8). After incubation at 37°C only saw morphotype was found (Table 1). Appearance of characteristic morphotypes i.e. extracellular matrix component synthesis was related with temperature incubation.

Table 1. Morphotypes and biofilm phenotypes on air-liquid interface of referent strain *S*. Enteritidis (SE1) and 14 isolates (SE2-SE15) at 25°C and 37°C

Isolates	Morphotype (25°C)[a]	Pellicle on air-liquid interface (25°C)[b]	Morphotype (37°C)[a]	Pellicle on air-liquid interface (37°C)[b]
SE 1.	bdar	++	saw	+
SE 2.	bdar	++	saw	+
SE 3.	saw	+	saw	+
SE 4.	bdar	++	saw	+
SE 5.	rdar	+++	saw	++
SE 6.	bdar	++	saw	+
SE 7.	rdar	+++	saw	++
SE 8.	saw	+	saw	-
SE 9.	bdar	++	saw	+
SE 10.	bdar	++	saw	+
SE 11.	rdar	+++	saw	+
SE 12.	rdar	+++	saw	+
SE 13.	rdar	+++	saw	+
SE 14.	rdar	+++	saw	++
SE 15.	rdar	+++	saw	++

[a] morphotypes on Congo Red agar (rdar) red, dry and rough, (bdar) brown, dry and rough and (saw) smooth and white; [b] phenotype on air-liquid interface: (-) the complete absence of pellicle - lack of ability to produce biofilm; (+) formation of very thin pellicle - weak biofilm producer; (++) formation of thin pellicle - moderate biofilm producer; (+++) formation of strong and thick pellicle - strong biofilm producer

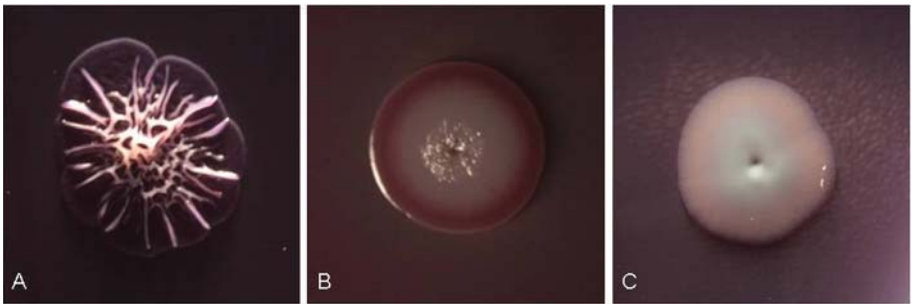


Figure 1. Morphotypes of *S*. Enteritidis after 96h incubation at 25°C on Congo Red LB agar w/o NaCl. A) red, dry and rough (rdar), B) brown, dry and rough (bdar) and C) smooth and white (saw) morphotype

Biofilm at the air-liquid interface

Obtained results for biofilm phenotype at the air-liquid interface i.e. ability to form a pellicle at the air-liquid interface at 25°C and 37°C after 96h of incubation are presented in Table 1.

As shown in Table 1. within 96h of incubation at 25°C the formation of a strong and thick pellicle (+++) was observed in seven isolates (SE5, SE7, SE11-SE15). In 5 isolates (SE2, SE4, SE6, SE9, and SE10) and the referent strain (SE1) the formation of a thin pellicle (++) was observed. Isolates SE3 and SE8 form a very thin pellicle (+).

Most of the isolates at 37°C had a weak production of these biofilm phenotypes (+), with the exception of isolates SE5, SE7, SE14 and SE15 in which a thin fragile pellicle (++) was formed, prone to rapid disintegration. Disintegration lags behind a very small ring of bacteria maintained on the glass at the air–liquid interface. At this temperature only one isolate (SE8) did not form a pellicle at the air–liquid interface.

Crystal violet assay

The biofilm forming ability of 14 isolates *S*. Enteritidis and the reference strain was determined at temperatures 25°C and 37°C using the Crystal violet assay.

The obtained ΔOD_{595} values under different temperatures of incubation for all tested isolates (SE2–SE15) and referent strain (SE1) are shown in Fig. 2. and 3. All tested isolates including the reference strain were capable of biofilm production on polystyrene microtiter plates after 48h incubation at both tested temperatures, but to various extents. On the basis of the obtained ΔOD_{595} values at 25°C isolates were classified into three categories - strong, moderate and weak biofilm producers, as previously described by Stepanović *et al.,* [31] The cut-off point ODc for the biofilm production was set at 0.125. At this temperature 7 isolates (SE5, SE7, SE11-SE15) were classified as strong biofilm producers with ΔOD_{595} that ranged from 0.502 (SE11) to 1.119 (SE5). Five isolates (SE2, SE4, SE6, SE9 and SE10) and the referent strain

SE1 were classified as moderate biofilm producers with ΔOD_{595} value that ranged from 0.290 (SE2) to 0.484 (SE6). In two isolates, SE3 and SE8, obtained ΔOD_{595} was <2ODc which classified them as weak biofilm producers.

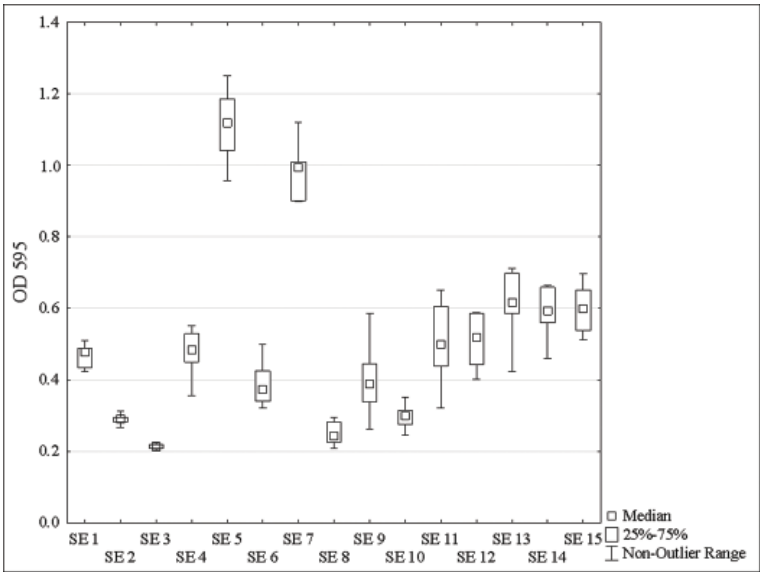


Figure 2. Biofilm formation of referent strain *S*. Enteritidis (SE1) and 14 isolates (SE2-SE15) at 25°C. Results are represented as median value of sixplicates. Boxes represent interquartile range (IQR) from the 25th (Q1) to 75th (Q3) percentile. Within boxes medians are indicated as individual data points (□). Whiskers extend below and above the box range, from the lowest to the highest values, respectively

According to the obtained values at 37°C the tested isolates were classified into two categories - moderate and weak biofilm producers. Within isolates categorized as moderate biofilm producers (SE4, SE5, SE6, SE7, SE9, SE14 and SE15) and referent strain (SE1) obtained values of ΔOD_{595} ranged from 0.263 (SE6) to 0.472 (SE7). ΔOD_{595} determined for all other isolates (SE2, SE3, SE8, SE10, SE11, SE12 and SE13) was <2ODc which classified them as weak biofilm producers. Obtained ΔOD_{595} values of these isolates ranged from 0.156 (SE3) to 0.239 (SE2). At temperature incubation of 37°C none of the tested isolates was assessed as a strong biofilm producer.

Statistical analysis of biofilm quantities after 48h of incubation showed statistically higher values at 25°C in comparison to 37°C ($p<0.05$). Comparing the obtained results of the applied assays, the existence of connection between the assays conducted at 25°C was ascertained (Fig. 4). In the case of assays conducted at 37°C the connection was not established.

Based on obtained characteristic of morphotypes and biofilm production, isolate SE5 was selected for the subsequent studies using CLSM.

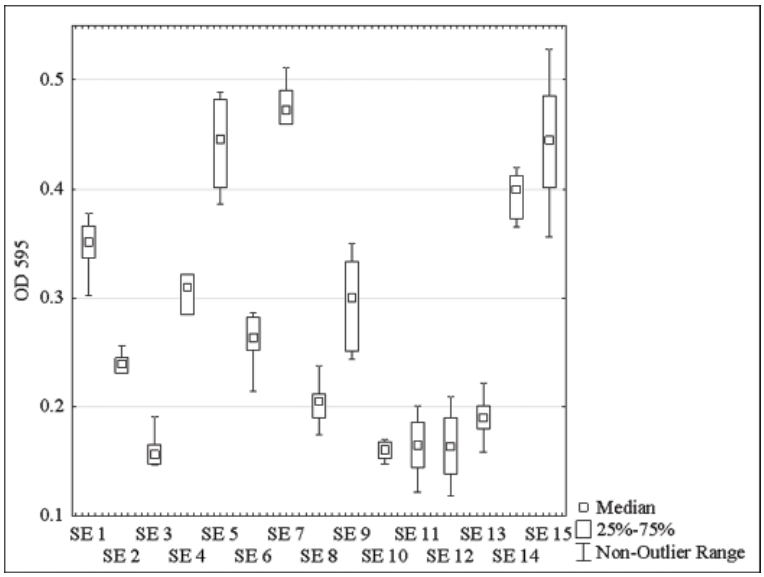


Figure 3. Biofilm formation of referent strain *S*. Enteritidis (SE1) and 14 isolates (SE2-SE15) at 37°C. Boxes represent interquartile range (IQR) from the 25th (Q1) to 75th (Q3) percentile. Within boxes medians are indicated as individual data points (□). Whiskers extend below and above the box range, from the lowest to the highest values, respectively

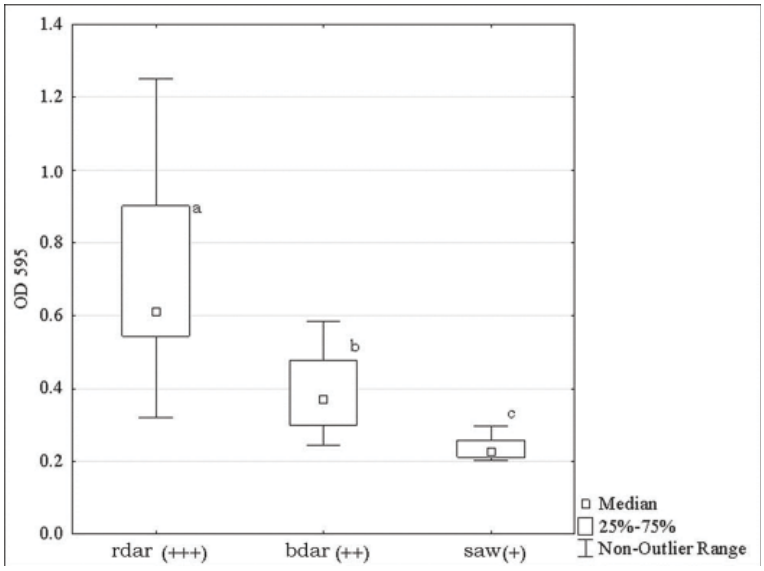


Figure 4. Comparison between the applied assays (Morphotype, Biofilm in air – liquid interface and Crystal violet assay) at 25°C. Boxes represent interquartile range (IQR) from the 25th (Q1) to 75th (Q3) percentile. Within boxes medians are indicated as individual data points (□). Whiskers extend below and above the box range, from the lowest to the highest values, respectively. ab, ac - ($p<0,001$); bc - ($p<0,01$)

Confocal laser scanning microscopy (CLSM)

Results on the visualization of the observed preformed biofilms using CLSM are presented in the form of sets of micrographs in Fig. 5-8. In particular, a CLSM micrograph illustrates the adherention ability of *S*. Enteritidis previously characterized as rdar morphotype (SE5) on glass surface after 24 and 48 hours. SE_{rdar} morphotype on submerged glass coupons in TSB formed biofilms with an unequal coverage and confluent growth areas where the bacteria formed clumps. After the initial 24 h of SE_{rdar} biofilm development, adherent cells were characterized by a mainly diffuse arrangement with the presence of small cell aggregations (Fig. 5, panels A and B). Using section views at higher magnification we identified differences in cell dimension. At this stage (after a period of 24h) cells were elongated (3-6μm), and there was a significant number of cells with compromised membranes (red cells). After 48h cells showed a slightly higher degree of adherence with the formation of larger and pronounced cell aggregations that were corresponded to form microcolonies Fig. 5, (panels C and D). Within this period the length of the cells was shorter and amounted 2.0 to 2.5μm.

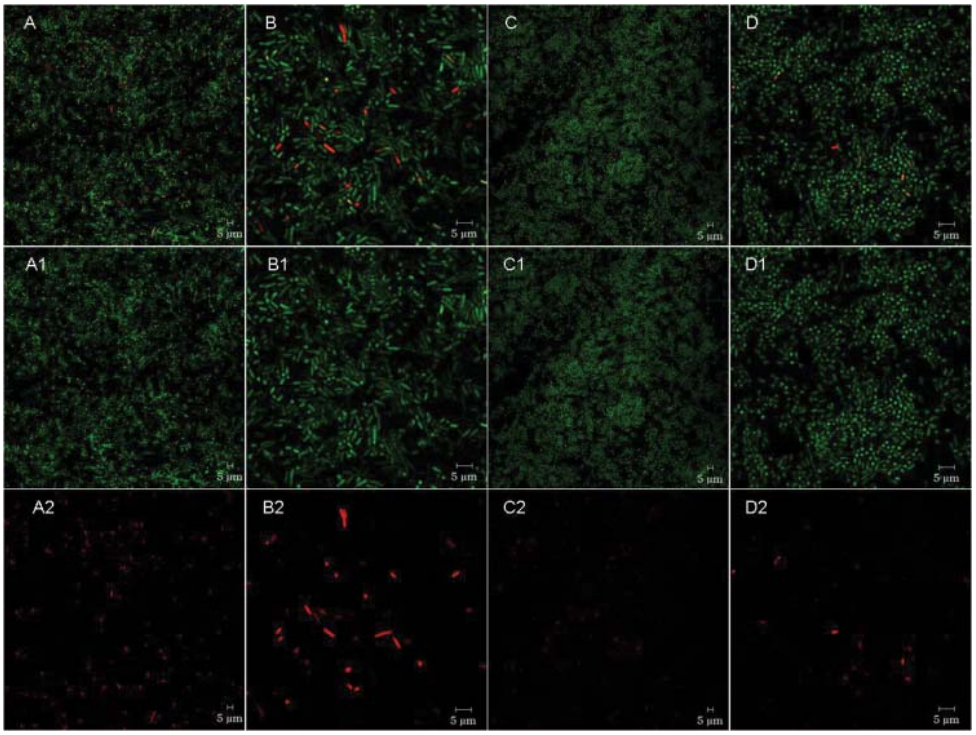


Figure 5. CLSM images of biofilm development by SE_{rdar} on glass coupons submerged in TSB. The biofilms were incubated for 24 hour (panels A and B) and for 48 hour (panels C and D). Image represented on panel A and C has a field size of 230.3 μm×230.3 μm (scan zoom 1); on panels B and D 76.8 μm x76.8 μm, (scan zoom 3) Color allocation: green = live cells (FDA); red = dead cells (PI) ABCD - with merging red and green channels, $A_1B_1C_1D_1$ - green channel, $A_2B_2C_2D_2$ - red channel. Scale bar represents 5μm.

As it can be seen on Fig. 6. B and D, Z projection the thicknesses of SE_{rdar} biofilms thus varied considerably with location. On the basis of the data obtained from Z projection of 0.97μm that were collected from the complete thickness of the biofilms in this study we measured a slightly higher maximal thickness at 24h old biofilm (15.58 μm) in regard to 48h old biofilm (13.64μm). Using the color coded depth map (3D-DepthCod), presented at Fig. 7, we have confirmed the lack of homogeneity in the thickness of the 24 hours-old biofilm, which varied in the range from 4μm to 12μm. The major part of the area on the map of 24h hours-old biofilm is presented as bright blue and green, corresponding to the thickness of 4μm to 10μm, while the areas that represent a thickness <4μm (blue) and >10μm (yellow-red) are smaller. On maps of 48 hours-old biofilms, the largest part of the surface is presented as a green to yellow-colored, corresponding to the thickness of 6μm - 10μm. We have also recorded the presence of thicker layers (red), but to a lesser extent. Based on these maps, it may be concluded that, in addition to homogeneity, there is no general difference between the thickness of the 24 and 48 hours-old biofilms, which is confirmed by the three-dimensional reconstruction, obtained from confocal stack images presented in Fig. 8. Images obtained by CLSM represent the general trend seen in three independent experiments.

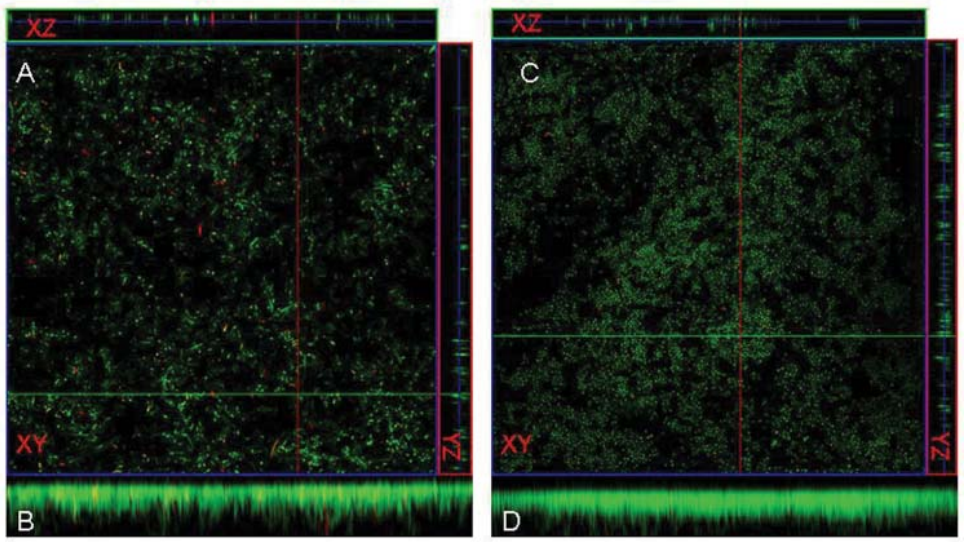


Figure 6. Orthogonal projection (XY, XZ, i YZ) of preformed biofilms by SE_{rdar} on glass coupons submerged in TSB for 24 hours (A and B) and for 48 hours (C and D). The green line on the XY image shows where the stack is cut to form the XZ image. The red line on the XY image shows where the stack is cut to form the YZ image (AC). The blue line on the XZ and YZ images shows the location of the XY slice. Z projection (BD)

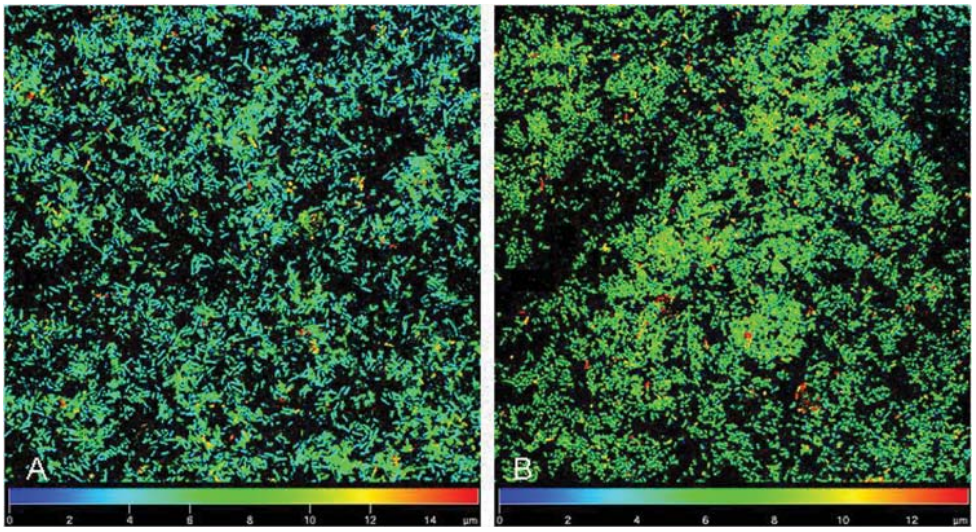


Figure 7. Color coded depth map (DepthCod) of biofilm development by SE_{rdar} on glass coupons submerged in TSB. The biofilms were incubated for 24 hours (A and B) and for 48 hours (C and D)

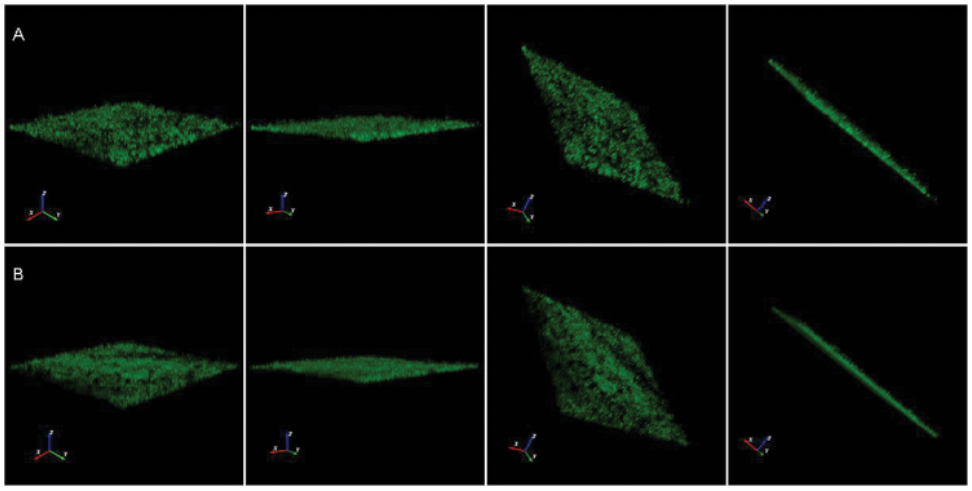


Figure 8. Three-dimensional reconstructions of live cells in biofilm by SE_{rdar} on glass coupons submerged in TSB. The biofilms were incubated for 24 hours (panel A) and for 48 hours (panel B). For each image the position axis are given in the bottom left corner

DISCUSSION

Since several authors have described a correlation between phenotypic appearance on Congo Red agar and biofilm formation [18,36] as well as biofilm phenotype at the air-liquid interface and biofilm formation [22,30] in *Salmonella* serotyps, assays applied in the present study were used as a phenotypic criterion for the characterization of

isolates. Both biofilm phenotypes are correlated with the production of extracellular matrix components.

Results in Table 1 show that the appearance of rdar morphotype and the formation of a strong and thick pellicle (+++) can be observed only at incubation temperature of 25°C. It has been already appointed that the synthesis of matrix components (curli and cellulose) is under the control of a complex regulatory system and depends on the expression of transcriptional regulator csgD, which regulates the production of both components of the biofilm matrix [36]. Furthermore, researches revealed that variability of external factors such as temperature, pH, osmolality, availability of nutrients and oxygen, may affect the expression of the transcriptional regulator csgD, and consequently the production of the matrix components. CsgD transcription, and thereafter the production of the matrix components curli and cellulose, is maximal under temperatures below 30°C, low osmolality, limited availability of nutrients and aerobic conditions [36,37], which is in accordance with the obtained results. Figure 9. demonstrates the strong temperature dependence of specific morphotype expression, i.e. synthesis of biofilm matrix components.

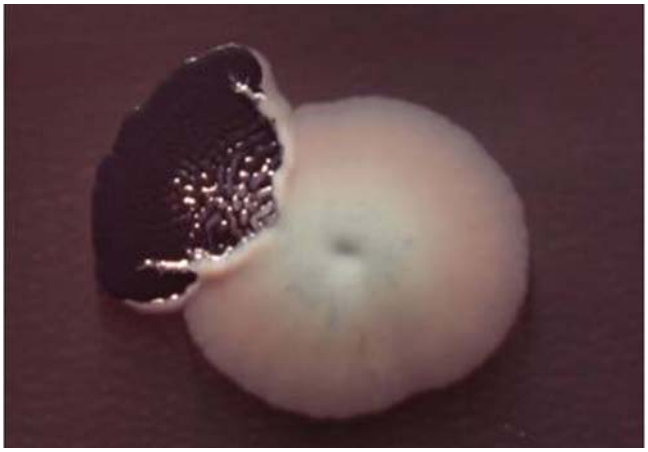


Figure 9. Transformation saw morphotype to rdar. This phenomenon occurred as a consequence of the decrease of temperature incubation from 37°C to 25°C within 24 hours

The presented picture (Fig 9.) has been taken during the current study and shows the transformation from smooth to rough colony, caused by the change of temperature during incubation. This transformation, occurred when the incubation temperature of 37°C was abruptly decreased to 25°C, is in accordance with the fact that a few different sets of genes, controlled by transcriptional regulator csgD, have been activated when the temperature was below 30°C. Among natural isolates of *Salmonella* spp., synthesis of biofilm matrix components and expression of rdar morphotypes are limited to lower temperatures, however, researches revealed that biogenesis of curli may occur even at 37°C, but only under iron starvation conditions. Also, it has been revealed that

the presence of individual point mutations in csgD region may result in the expression of this morphotype, irrespective of temperature [38].

Observing the results of the biofilm quantification, showed in Fig 2 and Fig 3, it was indicated that more biomass was formed at 25°C. This observation is in accordance with the findings of Stepanović *et al.* [31]. The named authors investigated the influence of three different incubation temperatures (~ 22°C, 30°C and 37°C) on biofilm formation by 30 isolates of *S. enterica* (*S.* Enteritidis n=29 and *S.* Typhimurium n=1) using TSB, and they reported that biofilm formation occurred under all tested temperature conditions, but the highest biofilm yield was obtained after 48 hours of incubation at ~ 22°C. Higher biofilm yield at close to ambiental temperatures was also achieved in previous investigations [28, 39].

By observing OD values obtained under conditions of both tested temperatures (Fig 2 and Fig 3), it can be concluded that tested isolates vary in the ability to produce biofilms. SE isolates tested in this research were classified in three categories by their biofilm-forming ability – strong, moderate and weak biofilm producers, which indicate that biofilm-forming ability is strain-dependent. Strain variability of biofilm-forming ability of serotype Enteritidis has been also confirmed in investigations by Stepanović *et al.* [31]. Within these investigations 30 isolates (29 of serotype Enteritidis) were tested, and after 48 hours of incubation at all tested temperatures (~ 22°C, 30°C and 37 °C) the isolates were categorized as strong, moderate and weak biofilm-producers and those that did not produce biofilm at all. Comparing the results of the applied assays, the existence of a connection between the assays conducted at 25°C was ascertained.

Isolates which expressed a specific morphotype on Congo Red agar, at the air-liquid interface formed a related type of pellicle: rdar morphotype (+++), bdar morphotype (++) and saw morphotype (+). The obtained results are in accordance with results of Solano, et al. [22], who tested the biofilm phenotype of 204 *S.* Enteritidis isolates at the air-liquid interface reported that 71% of isolates had the ability to form this biofilm-phenotype in Luria-Bertani broth after 96 hours of incubation at 28°C. Most of these isolates express rdar morphotype (93%). Three isolates at the air-liquid interface formed very thin unstable pellicles, correlated with bdar morphotype. Total of 27.5% isolates showed complete absence of pellicles and all these isolates on Congo Red agar formed saw morphotype. Furthermore, comparing OD values (results obtained in CV assay) of groups of isolates, each representing one of the three detected morphotypes (rdar, bdar and saw), statistically significant differences between these three groups were established (Fig 4).

The correlation between specific morphotype and biofilm-forming ability on microtitar plates after 96 hours incubation at 28°C was confirmed in reports by Malcova *et al.* [29]. Opposite to this research, Solomon *et al.,* [27] have not established a statistically significant correlation between OD values of isolates representing morphotypes rdar, bdar and saw when they were grown in TSB at 30°C. They detected a significant correlation when isolates were grown in poor nutrient media, ($TSB_{1/20}$), but only

between groups representing rdar and bdar morphotypes and the group representing saw morphotype. Difference between OD values obtained for isolates representing rdar and bdar morphotypes was not statistically significant even then. Mismatch between the mentioned results and results obtained within this research may be caused by short incubation time (24h), but also by incubation temperature (30°C). Regarding biofilm-formation, this temperature can be observed as critical, because synthesis of the main matrix components occurs at temperatures below 30°C.

As in the case of morphotypes, a statistically significant difference was established between intensity of pellicle production (+++, ++, +) and absorbance values obtained by CV assay in microtitar plates. Isolates reported to form a strong and thick pellicle (+++) were characterized by higher absorbance values in CV assay, comparing to isolates that form thin or very thin pellicle. Similar results were reported by Vestby *et al.* [30]. In the research of Solano *et al.* [22], a strong correlation between pellicle production and biofilm-forming ability on polyvinyl chloride surface was detected, opposite to research of Karaca *et al.* [28] who established a negative correlation between these tests.

In the case of assays conducted at 37°C, a connection was not established, because at this temperature on Congo Red agar only saw morphotype was detected, and this morphotype is characterized by a lack of synthesis of matrix components. Tests conducted in liquid media showed adherence from weak to moderate biofilm production. This finding at 37°C can be explained by the fact that near 30% of *Salmonella* spp. genome has been regulated differently in agar and broth systems [37]. Occurrence of saw morphotype at this temperature is most likely the consequence of deactivation of the csgD transcriptional regulator, while some adherence obtained in tests in liquid media could be explained by activation of different sets of genes that enable the synthesis of fimbria type 1 which requires a temperature of 37°C. Besides, capsular polysaccharides possibly contribute to higher adherence, because their synthesis occurs irrespective of temperature.

Presented micrographs (Figs 5-8) represent structures of 24 and 48h old biofilms of SE_{rdar} isolates on glass surfaces. From the micrographs it can be noticed that the initial phase of biofilm formation accompanied by slight cell aggregation occurs after 24 hours, while more intensive cell aggregation followed by formation of micro colonies occurs after 48 hours. This finding is in accordance with results of Wang *et al.* [40], who revealed similar observations analyzing the biofilm-forming ability of a cocktail of six *Salmonella* strains (*S*. Typhimurium, *S*. Agona, *S*. Heidelberg, *S*. Derby, *S*. Indiana, and *S*. Infantis), in TSB, on the surface of stainless steel. Bridier *et al.* [41] investigated the architecture of biofilms in polystyrene microtitar plates, formed by nine different strains of *S.enterica* (one isolate belonged to serotype Enteritidis), and their results indicate that these strains formed only a few, small scattered cell clusters. In the mentioned studies the biofilm-forming ability has not been related with morphotypes. Structure of the formed biofilm of SE_{rdar} corresponds to the structures of monoculture biofilms formed in static conditions.

CONCLUSION

Presented results revealed that the biofilm forming ability can be assumed from morphotypes obtained on Congo Red agar. Within this research, the ability of SE isolates to colonize surfaces was demonstrated, particularly at ambient temperatures, which are common in food processing facilities. Therefore, future investigation should be based on establishing connections between the biofilm forming ability and persistence and cross contamination under real industrial conditions.

Based on the obtained results, it can be reported that the occurrence of rdar morphotype among SE isolates is quite common. Presented micrographs are a valuable scientific contribution to complementing knowledge about the biofilm structure formed by rdar morphotype of *S*. Enteritidis.

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SPOSOBNOST FORMIRANJA BIOFILMA *SALMONELLA* ENTERITIDIS *IN VITRO*

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Salmonella enterica serotip Enteritidis je važan alimentarni patogen, a zbog sposobnosti formiranja biofilma koja je ustanovljena kod velikog broja različitih izolata, danas mu se pridaje sve veći značaj. Kvalitativna ispitivanja sposobnosti formiranja biofilma su izvedena određivanjem morfotipa kolonija na Kongo crvenom agaru i primenom pelikula testa, dok su kvantitativna ispitivanja izvedena primenom kristal violet testa na mikrotitar pločama. Testovi sposobnosti produkcije biofilma su izvedeni sa 14 izolata *Salmonella* Enteritidis, a kao referentni soj korišćen je *Salmonella* Enteritidis ATCC 13076.

Na osnovu morfotipa kolonija na Kongo red agaru na temperaturi inkubiranja od 25°C među testiranim izolatima detektovana su tri morfotipa rdar (red, dry and rough), bdar (brown dry and rough) i saw (smooth and white). Polovina testiranih izolata je pokazivala rdar morfotip. Izolati koji su pokazali karakterističan morfotip na ovoj temperaturi su formirali na vazduh tečnost međufazi isti tip pelikule.

Osim toga, upoređivanjem vrednosti apsorbanci izmerenih u kristal violet testu između grupa izolata koji reprezentuju jedan od tri detektovana morfotipa (rdar, bdar i saw) ustanovljeno je postojanje statistički značajnih razlika. Na temperaturi inkubiranja od 37°C detektovan je samo jedan morfotip (saw). Na osnovu vrednosti apsorbanci izmerenih primenom kristal violet testa na obe temperature inkubacije, izolati su na osnovu sposobnosti produkcije biofilma klasifikovani u tri kategorije - jaki, umereni i slabi biofilm. Pored toga su ustanovljene statistički značajne razlike u količini formiranog biofilma nakon 48h ($P<0.05$) u odnosu na temperature inkubiranja 25°C i 37°C. U istraživanju su prikazane mikrografije i rekonstrukcija trodimenzionalne strukture razvojnih faza biofilma izolata rdar morfotipa na površini stakla upotrebom konfokalne laserske skening mikroskopije.