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Preliminary communication

THE EFFECTS OF ENVIRONMENTAL FACTORS ON PLANKTONIC GROWTH AND BIOFILM FORMATION OF *SERRATIA ODORIFERA* AND *SERRATIA MARCESCENS* ISOLATED FROM TRADITIONALLY MADE CHEESE

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In this study, the effects of different temperature, pH, salt and glucose concentrations on the planktonic growth, biofilm formation, and formed biofilm of *Serratia odorifera* and *Serratia marcescens,* isolated from traditionally made cheese, were investigated using spectrophotometric method. The investigated strains demonstrated best planktonic growth and biofilm formation in Tryptic soy broth. The limiting factors for the planktonic growth and biofilm formation were temperature below 4 °C and salt concentration above 4%. Temperature of 37 °C and 44 °C, as well as various concentrations of glucose, stimulated the planktonic growth of bacteria. Moderate influence on biofilm formation was demonstrated at 37 °C as well as at various concentrations of glucose. These results were in accordance with the origin of bacteria, since the isolates were obtained from cheese.

Keywords: biofilm formation, environmental factors, planktonic growth, *Serratia*

Bacteria from the *Enterobacteriaceae* family may affect the quality of food (usually milk and cheese) by their metabolism, and they might propagate during maturity process (Chaves-Lopez et al., 2005). The ability of enterobacteria (*Enterobacter, Serratia, Escherichia, Hafnia, Citrobacter*, and *Klebsiella*), isolated from cheese, to produce acid and biogenic amines was examined by a few researchers. Marino and co-workers (2000) found a positive correlation between the concentration of cadaverine and the number of enterobacteria.

Serratia marcescens is Gram-negative bacterium, which is able to populate a wide variety of ecological niches (Grimont et al., 1977). According to Aucken and Pitt (1998), it is an opportunistic human pathogen responsible for many infections and resistant to antibiotics. *Serratia odorifera* was identified in a local Italian cheese (Chaves-Lopez et al., 2005).

Environmental factors (temperature, sugar, salt, pH, and nutrients) present in foods and food-processing environments play significant role in adhesion and biofilm formation (Mirkar et al., 2016). According to Khangholi and Jamalli (2016), one way of bacterial adaptation to the environmental conditions is the ability to form biofilm. The capability of *S. marcescens* to cause infections and survive in the environment is attributed to its ability to form biofilm (Kalivoda et al., 2010). Bacteria regulate gene expression in response to different environmental signals, such as temperature, oxygen and carbon dioxide concentrations, pH, and nutrient availability (Harjai et al., 2005).

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The aims of this study were investigation of the planktonic growth and ability to form biofilm of *S. odorifera* and *S. marcescens* biogp 1 in two different broths, under the influence of different temperatures, pH, concentrations of NaCl and glucose, as well as the impact of the mentioned environmental factors on the formed biofilm.

1. Materials and methods

1.1. Strains and growth conditions

For the tests in this study, strains *S. odorifera* KGPMF 18 and *S. marcescens* biogp 1 KGPMF 19 were used. The bacteria were previously isolated from traditionally made Serbian cheese (Sokobanja region) and identified at the Laboratory for Microbiology at the Faculty of Science, University of Kragujevac (KGPMF) (Mladenović et al., 2018). The collection of identified bacterial species was stored in a 20% glycerol/medium mixture at –80 °C.

1.2. Influence of temperature, pH, salt and glucose concentrations on planktonic growth in TSB and MH media

The examination of the effect of temperature (4 °C, 37 °C, 44 °C) on the growth of *Serratia* sp. was conducted on Tryptic soy broth (TSB) and Muller-Hinton broth (MH), standard or modified compositions.

To study the effect of pH, media with different pH values (5.5, 6.5, 7, 7.5, 8.5) were prepared. The pH was set to 5.5, 6.5, and 7 with addition of HCl and to 7.5 and 8.5 with addition of NaOH. For TSB growth pH 7.5 was used as control, while for growth in MH, control was set to pH 7.

Both tested media were modified with the addition of NaCl (4%, 6.5%, and 8%) in order to investigate the effects of different salt concentrations. Growth in TSB with 4% NaCl and in pure MH served as controls.

The effect of different concentrations of glucose (0.5%, 1.5%, 2.5%, and 3.5%) was investigated in modified TSB and MH media. Growth in TSB with 0.25% of glucose and in pure MH served as controls.

Initial bacterial suspension of 10 µl (10^8–10^9 CFU ml^{-1}) was added to 3 ml of each type of medium. Samples were incubated for 24 h. Sterility controls were pure TSB and MH media. Results were determined with spectrophotometer at 600 nm. Each experiment was performed in triplicate.

1.3. The determination of antibiofilm activity

1.3.1. Pellicle test. The ability to form a biofilm phenotype or pellicle formation on the air-liquid interphase was determined using pellicle assay according to the method of Vestby and co-workers (2009), with modifications. TSB and MH media of 1.8 ml were inoculated with 0.2 ml of initial bacterial suspension (10^8–10^9 CFU ml^{-1}) and then incubated for 96 h at 37 °C. Categorization of isolates and their ability to produce biofilm were based on the production of pellicle on the surface of the liquid phase according to the following scheme: solid fat formed pellicle (+++) – good biofilm producer; thin pellicle formed (++) – moderate biofilm producer; very thin pellicle (+) – weak biofilm producer; complete absence of pellicle (-) – no biofilm production. Pellicle test was repeated three times for each strain.

1.3.2. Biofilm formation assay and quantification. The ability of *S. odorifera* KGPMF 1 and *S. marcescens* biogp 1 KGPMF 19 to form biofilm was assayed according to O'Toole and Kolter (1998), with some modifications.

In sterile 96-well tissue culture plates (Sarstedt, Germany) 100 µl TSB or MH broth (with different pH, salt and glucose concentrations) and 10 µl of fresh bacterial suspension (1.0 McFarland) was added to each well. After incubation at 4 °C, 37 °C, and 44 °C for 48 h, the content of each well was gently removed by tapping the plates. The rest of the experiment was done as described by Muruzović and co-workers (2016).

1.3.3. The effect of temperature, pH, salt and glucose concentrations on formed biofilm. The tissue culture 96-well microtiter plates (Sarstedt, Germany) were prepared by adding 100 µl TSB or MH broth and 10 µl of fresh bacterial suspension (1.0 McFarland) to each well. The inoculated microtiter plates were incubated at 37 °C for 24 hours. After incubation, the content of each well was gently pulled out. Then, 100 µl TSB or MH broth with different pH, salt and glucose concentrations was added to each well, and the microtiter plates were incubated at 4 °C, 37 °C, and 44 °C for 24 hours. After incubation, the content of each well was gently removed by tapping the microtiter plates. After that, experiment was carried out as described by Muruzović and co-workers (2016).

1.4. Data analysis

All data were presented as means ± standard deviations using Microsoft Excel (Redmond, Washington, DC, USA). Paired *t*-test was used for statistical analysis of the results via IBM SPSS Statistics 20.

2. Results and discussion

Tested bacteria were incubated in different media at three temperatures. After incubation, there was no growth at 4 °C. Planktonic growth at 37 °C in TSB was statistically significantly higher compared to growth in MH ($P<0.05$). The differences between planktonic growth in TSB and MH at 44 °C were statistically not significant ($P>0.05$) (Tables 1, 2, and 3).

2.1. Influence of different temperature, pH, and different concentrations of salt and glucose on the planktonic growth in TSB and MH media

All tested pH values were limiting factor for planktonic growth of *S. odorifera* and *S. marcescens* in TSB at 37 °C and 44 °C, except for pH 8.5.

All tested pH values were limiting factor for planktonic growth of *S. odorifera* and *S. marcescens* in MH at 37 °C, except for pH 6.5 in case of *S. marcescens*, where the growth was stimulated. At 44 °C, the results were similar. Based on the results, it can be concluded that the bacterial growth was better at the 37 °C than at 44 °C at all tested pH (Table 1).

All concentrations of salts reduced the growth of *S. odorifera* and *S. marcescens* in both media at 37 °C and 44 °C (Table 2).

In TSB with different concentrations of glucose at 37 °C and 44 °C, both species demonstrated lower growth compared to control. In MH with glucose at 37 °C, *S. odorifera* showed better growth than in control. *S. marcescens* growth was equal that of the control for each glucose concentrations, except for 2.5%, where growth was lower. At 44 °C, both species demonstrated better growth in the presence of glucose than in control (Table 3).

Table 1. The effect of pH on planktonic growth

Species	TSB at 37 °C					TSB at 44 °C				
	5.5	6.5	7	7.5*	8.5	5.5	6.5	7	7.5*	8.5
S. odorifera KGPMF18	0.02±0.00	0.86±0.02	1.81±0.00	1.67±0.00	1.67±0.00	0.02±0.00	0.88±0.22	1.03±0.01	1.42±0.02	1.22±0.01
S. marcescens biogp1 KGPMF 19	0.01±0.00	0.71±0.03	0.76±0.01	1.58±0.03	1.51±0.02	0.01±0.00	0.53±0.18	0.67±0.03	1.30±0.24	1.30±0.11
	MH at 37 °C					MH at 44 °C				
	5.5	6.5	7*	7.5	8.5	5.5	6.5	7*	7.5	8.5
S. odorifera KGPMF18	0.05±0.00	0.60±0.01	0.73±0.00	0.69±0.04	0.41±0.06	0.02±0.00	0.24±0.02	0.21±0.03	0.19±0.04	0.11±0.03
S. marcescens biogp1 KGPMF 19	0.07±0.01	0.80±0.01	0.75±0.09	0.72±0.07	0.43±0.01	0.02±0.00	0.38±0.05	0.30±0.05	0.23±0.00	0.17±0.00

Values are presented as mean ± standard deviation of absorbance measured at 600 nm;
*: growth control

Table 2. The effect of NaCl concentration on planktonic growth

	% of NaCl in TSB at 37 °C			% of NaCl in TSB at 44 °C		
	4%	6.5%	8%	4%	6.5%	8%
S. odorifera KGPMF18	1.41±0.01	0.50±0.04	0.16±0.00	0.92±0.03	0.47±0.27	0.09±0.03
S. marcescens biogp1 KGPMF 19	0.84±0.00	0.62±0.01	0.49±0.02	0.72±0.02	0.50±0.31	0.37±0.23
	% of NaCl in MH at 37 °C			% of NaCl in MH at 44 °C		
	4%	6.5%	8%	4%	6.5%	8%
S. odorifera KGPMF18	0.25±0.02	0.08±0.00	0.04±0.02	0.06±0.00	0.02±0.00	n.g.
S. marcescens biogp1 KGPMF 19	0.25±0.01	0.1±0.01	0.02±0.00	0.07±0.03	0.04±0.00	n.g.

Values are presented as mean ± standard deviation of absorbance measured at 600 nm
n.g.: no growth

Table 3. The effect of glucose concentration on planktonic growth

Species	% of glucose in TSB at 37 °C					% of glucose in TSB at 44 °C				
	Growth control	0.5%	1.5%	2.5%	3.5%	Growth control	0.5%	1.5%	2.5%*	3.5%
S. odorifera KGPMF18	1.67±0.00	1.31±0.04	1.07±0.11	1.09±0.04	0.96±0.14	1.42±0.02	1.25±0.09	1.15±0.29	1.08±0.04	0.97±0.09
S. marcescens biogp1 KGPMF 19	1.58±0.03	0.90±0.23	0.86±0.22	0.77±0.01	0.65±0.01	1.30±0.24	0.84±0.07	0.68±0.04	0.57±0.06	0.47±0.00
	% of glucose in MH at 37 °C					% of glucose in MH at 44 °C				
	Growth control	0.5%	1.5%	2.5%	3.5%	Growth control	0.5%	1.5%	2.5%	3.5%
S. odorifera KGPMF18	0.73±0.00	0.81±0.02	0.72±0.02	0.87±0.01	0.81±0.05	0.21±0.03	0.60±0.01	0.60±0.03	0.61±0.03	0.63±0.02
S. marcescens biogp1 KGPMF 19	0.75±0.09	0.76±0.04	0.73±0.02	0.48±0.05	0.74±0.02	0.30±0.05	0.61±0.14	0.65±0.10	0.49±0.01	0.69±0.09

Values are presented as mean ± standard deviation of absorbance measured at 600 nm

Table 4. The influence of pH on the biofilm formation and formed biofilm in TSB and MH

	Biofilm formation in MH					Formed biofilm in MH				
	pH 5.5	pH 6.5	pH 7*	pH 7.5	pH 8.5	pH 5.5	pH 6.5	pH 7*	pH 7.5	pH 8.5
S. marcescens biogp 1 KGPMF 19	0.07±0.02	0.07±0.00	0.06±0.00	0.07±0.02	0.00±0.00	0.07±0.04	0.08±0.02	0.13±0.01	0.12±0.02	0.07±0.03
S. odorifera KGPMF 18	0.05±0.02	0.05±0.01	0.07±0.00	0.05±0.01	0.08±0.00	0.09±0.01	0.08±0.02	0.11±0.00	0.1±0.03	0.15±0.00
	Biofilm formation in TSB					Formed biofilm in TSB broth				
	pH 5.5	pH 6.5	pH 7	pH 7.5*	pH 8.5	pH 5.5	pH 6.5	pH 7	pH 7.5*	pH 8.5
S. marcescens biogp 1 KGPMF 19	0.08±0.04	0.3±0.05	0.28±0.04	0.31±0.08	0.19±0.02	0.24±0.03	0.36±0.03	0.28±0.01	0.32±0.04	0.26±0.00

Values are presented as mean ± standard deviation of absorbance measured at 630 nm
* growth control

S. odorifera was identified in semi-hard cheese of Portuguese origin (Kongo et al., 2008). *Serratia* sp. reaches food incidentally, during the process of production. In our study, it was noticed that temperature at 4 °C, low pH, and all concentrations of salt showed inhibitory effect on the planktonic growth of *Serratia* species in TSB and MH media.

2.2. Determination of antibiofilm activity

2.2.1. Pellicle test. According to the results, neither species were able to form pellicle in TSB or MH at 37 °C.

2.2.2. *The influence of different temperature, pH, and different concentrations of salt and glucose on biofilm formation and on biofilm formed in TSB and MH.* The biofilm formation ability of the strains was tested in different media at three temperatures. After incubation, it was noticed that none of them formed biofilm at 4 °C and 44 °C. It was also observed that *S. odorifera* had no ability to form biofilm in TSB.

According to the results, all tested pH values, except pH 7.5, were limiting factors for biofilm formation and on formed biofilm of *S. marcescens* grown in TSB (Table 4.).

In MH pH 8.5, biofilm formation of *S. odorifera* was stimulated, but biofilm formation of *S. marcescens* was reduced. The influence of pH on formed biofilm was manifested in the reduction of formed biofilm (Table 4).

TSB with different concentrations of salt showed reducing effect on biofilm formation of *S. marcescens*. The size of formed biofilm was reduced, too (Table 5).

MH with different concentrations of salts had reducing effect on the biofilm formation of both species. In case of *S. odorifera* 4% and 6.5% of salt showed stimulating effect on biofilm formation. The same concentrations of salt reduced the formed biofilm of *S. marcescens*, while concentration of 8% stimulated the further growth of biofilm (Table 5).

Table 5. Influence of NaCl on the biofilm formation and formed biofilm in TSB and MH

	Biofilm formation in MH			Formed biofilm in MH		
	4%	6.5%	8%	4%	6.5%	8%
S. marcescens biogp 1 KGPMF 19	0.04±0.01	0.01±0.00	0.003±0.00	0.05±0.02	0.12±0.02	0.15±0.02
S. odorifera KGPMF 18	0.06±0.01	0.02±0.00	0.002±0.00	0.12±0.01	0.12±0.00	0.11±0.00
	Biofilm formation in TSB			Formed biofilm in TSB		
	4%	6.5%	8%	4%	6.5%	8%
S. marcescens biogp 1 KGPMF 19	0.09±0.00	0.03±0.00	0.01±0.01	0.2±0.02	0.15±0.01	0.12±0.01

Values are presented as mean ± standard deviation of absorbance measured at 630 nm

TSB with 0.5% and 1.5% of glucose reduced the biofilm formation, while 2.5% and 3.5% showed stimulating effect on the biofilm formation of *S. marcescens*. Further growth of formed biofilm of *S. marcescens* was stimulated by 0.5% and 1.5% of glucose (Table 6).

MH with 0.5% of glucose stimulated biofilm formation of both bacteria, while other tested concentrations showed reducing effect. All tested concentrations of glucose demonstrated stimulating effect on already formed biofilm of *S. odorifera*, but on formed biofilm of *S. marcescens* they showed reducing effect. The only exception was the concentration of 3.5%, where further growth of formed biofilm was stimulated (Table 6).

Table 6. The influence of different concentrations of glucose on biofilm formation and formed biofilm in TSB and MH

	Biofilm formation in MH				Formed biofilm in MH			
	Growth control (0.25%)	0.5%	1.5%	2.5%	3.5%	Growth control	0.5%	1.5%	2.5%	3.5%
S. marcescens biogp 1 KGPMF 19	0.06±0.00	0.09±0.00	0.06±0.02	0.02±0.00	0.05±0.00	0.13±0.01	0.08±0.01	0.08±0.02	0.1±0.03	0.16±0.04
S. odorifera KGPMF 18	0.1±0.00	0.15±0.00	0.04±0.00	0.04±0.01	0.05±0.01	0.11±0.00	0.22±0.01	0.21±0.02	0.2±0.00	0.19±0.02
	Biofilm formation in TSB					Formed biofilm in TSB				
	Growth control (0.25%)	0.5%	1.5%	2.5%	3.5%	Growth control	0.5%	1.5%	2.5%	3.5%
S. marcescens biogp 1 KGPMF 19	0.31±0.08	0.25±0.00	0.23±0.04	0.51±0.03	0.34±0.09	0.32±0.04	0.36±0.02	0.43±0.09	0.32±0.06	0.33±0.04

Values are presented as mean ± standard deviation of absorbance measured at 630 nm

S. odorifera and *S. marcescens* showed no ability of pellicle formation in TSB and MH, but demonstrated ability of biofilm formation at 37 °C. According to Nandhagopal and Subashkumar (2016), biofilm formation of *S. marescens* at refrigeration temperature reached its maximum, compared to room temperature and 37 ºC. It has been confirmed that a temperature of 37 ºC can induce further biofilm development of *Enterococcus* species (Tendolkar et al., 2004). White-Ziegle and co-workers (2008) showed that biofilm was formed by *S. marescens* isolates at 23 ºC. According to Nandhagopal and Subashkumar (2016), biofilm formation of *Serratia* sp. at low temperature may have a role in the contamination of refrigerated foods. They also found that 1% NaCl decreased the biofilm formation, but 2% NaCl decreased the biofilm density. Studies showed that the increase of salt concentration, led to decrease of biofilm formation, up to a point when a higher concentration of salt did not affect further the growth of biofilm formed by enterococci (Asha et al., 2013). Our research showed that all concentrations of salt in TSB reduced the biofilm formation and also reduced the already formed biofilm of the *Serratia* species investigated. In MH, formed biofilm was stimulated at 8% of salt. Various concentrations of glucose showed stimulating or reducing effects on biofilm formation and formed biofilm. These results indicated that the presence of lactic acid bacteria in cheese affecting pH, can contribute to the control of number and presence of enterobacteria in cheese. Also, adding salt to cheese can prevent their growth. These are the possible mechanisms of cheese preservation.

3. Conclusions

The effects of pH, salt, and glucose at different temperatures on planktonic growth of *S. odorifera* and *S. marcescens* isolated from Sokobanja cheese were investigated for the first time in this paper. It was noticed that temperature at 4 °C, low pH, and all concentrations of salt showed inhibitory effect on the planktonic growth of *Serratia* species in both tested media. Glucose in TSB and MH showed stimulating effect on planktonic growth. Biofilm formation was possible only at 37 °C. These results contribute to better understanding the influence of environmental factors on the growth and development of bacteria. Further studies should be conducted to investigate other factors that could be used as preservatives in traditionally made cheeses.

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