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Ecological parameters which control the *in vitro* development and growth of *Klebsiella* isolates from traditional Serbian cheese

Ökologische Parameter, die in-vitro-Entwicklung und das Wachstum von Klebsiella-Isolaten aus traditionellem serbischem Käse steuern

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Summary

In this study, the effects of different temperatures, pH, salt concentrations and different growth media on the planktonic growth, biofilm formation, and formed biofilm of *Klebsiella oxytoca, Klebsiella ornithinolytica,* and *Klebsiella pneumoniae* were investigated. These bacteria were isolated from autochthonous Serbian cheese (from the Sokobanja region). *Klebsiella pneumoniae* ATCC 70063 was used as a positive control. The influence of environmental factors was examined. Planktonic growth, biofilm formation, and formed biofilm were determined using the spectrophotometric method. Bacterial strains demonstrated better planktonic growth and biofilm formation in Tryptic soy broth. The results of our study confirmed that limiting factors for planktonic growth and biofilm formation were the temperature of 4°C and the salt concentration above 6.5%. Lower development of biofilm was demonstrated in pH 5.5 and 8.5, as well as in the salt concentrations of 4% and 6.5% It can be concluded that various physicochemical parameters of natural processing conditions exerted substantial influence on the planktonic growth and biofilm formation of *Klebsiella* isolates originated from cheese.

Keywords: biofilm, cheese, *Klebsiella* spp, pH, salt concentration, temperature

Zusammenfassung

In dieser Studie wurden die Auswirkungen unterschiedlicher Temperaturen, pH-Werte, Salzkonzentrationen und unterschiedlicher Wachstumsmedien auf das Planktonwachstum, die Biofilmbildung und den gebildeten Biofilm von *Klebsiella oxytoca, Klebsiella ornithinolytica* und *Klebsiella pneumoniae* untersucht. Diese Bakterien wurden aus autochthonem serbischem Käse (aus der Region Sokobanja) isoliert. *Klebsiella pneumoniae* ATCC 70063 wurde als positive Kontrolle verwendet. Der Einfluss von Umweltfaktoren wurde untersucht. Das planktonische Wachstum, die Biofilmbildung und der gebildete Biofilm wurden unter Verwendung der spektrophotometrischen Methode bestimmt. Bakterienstämme zeigten ein besseres planktonisches Wachstum und eine bessere Biofilmbildung in tryptischer Sojabrühe. Die Ergebnisse unserer Untersuchung zeigten, dass begrenzende Faktoren für das Planktonwachstum und die Biofilmbildung die Temperatur bei 4 ° C und die Salzkonzentration über 6,5% waren. Eine geringere Entwicklung des Biofilms wurde bei pH 5,5 und 8,5 sowie bei Salzkonzentrationen von 4% und 6,5% nachgewiesen. Es kann geschlossen werden, dass verschiedene physikalisch-chemische Parameter der Verarbeitungsbedingungen in der Natur einen wesentlichen Einfluss auf das planktonische Wachstum und die Biofilmbildung von *Klebsiella*-Isolaten hatten aus Käse.

Schlüsselwörter: Biofilm, Käse, *Klebsiella* spp., PH-Wert, Salzkonzentration, Temperatur

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Introduction

The bacteria of the genera *Klebsiella* were frequently detected in a variety of environmental conditions, such as soil, vegetation, fermented food (traditionally made cheeses belong to this group) and water, including the drinking water distribution system (Podschun et al., 2001; Mladenović et al., 2018b). Species can often occur in soft cheeses made from unpasteurized milk. *Klebsiella* sp. were isolated from: Urfa cheese (Turkey) (Guven et al., 2008), Portuguese origin semi-hard cheese (*K. oxytoca*, *K. cloacae*, *K. pneumoniae*, *K. ornithinolytica*, *K. terrigena*) (Kongo et al., 2008), from cheese named ‚wara' in Nigeria (Ogbolu et al., 2014), Sokobanja cheese from Serbia (*Klebsiella oxytoca*, *K. ornithinolytica*, *K. pneumoniae*) etc. *Klebsiella* spp. is one of the pathogens capable of forming biofilm and that capability may be considered to be a virulence factor (Maldonado et al., 2007).

The environmental factors, including temperature, sugar, salt, pH, and nutrients that are present in foods and food-processing environments, play a significant role in adhesion and biofilm formation (Costa, 2014; Mirkar et al., 2016). The ability of bacteria to form biofilm is one way of adapting to environmental factors (Khangholi and Jamalli, 2016). It has been suggested that biofilm formation is a stress response of bacteria (Jefferson, 2004; Gandhi and Chikindas, 2007). Organized bacteria in biofilm are more resistant to antibiotics than planktonic bacteria (Melchior et al., 2006). In the food industry, food may become contaminated by pathogenic (undesirable bacteria) or non-pathogenic microorganisms. If the regulations related to sterilization and disinfection are not observed, the poor hygiene of the production processes is due to the appearance of undesirable bacteria (Costerton et al., 1995). The bacteria from the fam. Enterobacteriaceae may affect the quality of organic food (typically milk and cheese) and their number may grow throughout the maturity process of food (Chaves-Lopez et al., 2006). *Klebsiella* spp. belongs to the Enterobacteriaceae family. Enterobacteriaceae ferment a variety of carbohydrates, however, their ability to produce acid and gas from the fermentation of D-glucose is one characteristic that remains an important diagnostic property. Some members of the Enterobacteriaceae (*Klebsiella*) possess the ability to ferment lactose producing acid and gas. Enterobacteriaceae have been used for a long time as indicator organisms in the food industry (Cordier, 2006; Halkman and Halkman, 2014). It was demonstrated that the exposure of the cheese to 63°C for up to 15 min led to the complete destruction of *K. pneumoniae* and *Klebsiella oxytoca* (Massa et al., 1992). In another experiment, the heat resistance, and the temperature range (25,30,37,42°C) for the growth of *K. pneumoniae* in nutrient broth (EIKEN) were demonstrated (Tsuji et al., 1982). They also showed that the species did not survive 60°C. The bacterial growth of *K. oxytoca* ATCC 700324 was determined in pH levels between pH 5 and pH 8 (Erdogan-Yildirim et al., 2011).

Since the Sokobanja cheese (Southeastern Serbia) is produced in a traditional way (Mladenović et al., 2018b), the processing conditions allow the development of the members of fam. Enterobacteriaceae. The cheese was produced in countryside households around Sokobanja, southeast Serbia. Fresh, processed, and unpasteurized cow's milk was filtered after milking, and then heated to the temperature of 30–40ºC. A liquid rennet of microbiological origin based on chymosin was used for milk coagulation. The entire production of the cheese was carried out in wooden vessels. The quantity of salt added to the cheese was 6–8% of the cheese weight. *Klebsiella* spp. were isolated from this cheese (Mladenović et al., 2018b). Therefore, the aims of this study were to investigate the ability of planktonic growth and biofilm formation of *Klebsiella oxytoca* KGPMF 2, *K. oxytoca* KGPMF 4, *K. ornithinolytica* KGPMF 9 and *K. pneumoniae* KGPMF 11, isolated from above mentioned cheese in different broths and under the influence of various temperatures, pH and NaCl concentrations. Furthermore, the aim was to examine the influence of mentioned environmental factors on previously formed biofilm. The main goal of this research was to suggest optimal conditions that may negatively influence the development of the mentioned bacteria.

Material and methods

Bacterial strains

The effects of different temperatures, pH, salt concentrations and different growth media on planktonic growth and biofilm formation were tested against four bacteria isolated from Sokobanja cheese (Southeastern Serbia): *Klebsiella oxytoca* KGPMF 2, *K. oxytoca* KGPMF 4, *K. ornithinolytica* KGPMF 9 and *K. pneumoniae* KGPMF 11. The bacteria were previously isolated from autochthonous Serbian cheese (from Sokobanja region) and determined at the Laboratory for Microbiology, Faculty of Science, University of Kragujevac (Mladenović et al., 2018b). The collection of identified bacterial species was stored in a 20% glycerol/medium mixture at –80°C. *Klebsiella pneumoniae* ATCC 70063 was used as standard strain.

The assay for the effect of different temperatures, pH and NaCl concentrations on bacterial planktonic growth

The investigation of the influence of temperature on the growth of *Klebsiella* spp. was performed in Tryptic soy broth (TSB) and Muller-Hinton broth (MH) (Torlak, Belgrade, Serbia) of the standard or the modified composition. In 3 ml of each type of media, 10 µl of initial bacterial suspension was added. The turbidity of initial suspensions was adjusted using 0.5 McFarland densitometer (BioSan, Latvia). Initial bacterial suspensions contain about 10^8 colony forming units (CFU)/ml. All samples were prepared for each tested temperature (4°C, 37°C, 44°C). The samples were incubated for 24 h. These temperatures were selected since the temperature of 4°C was the temperature of storing cheese in the refrigerator, while the temperature of producing and preserving the Sokobanja cheese in the household was 20°C and the temperature for optimal bacterial growth in most cases was 37°C. Sterility controls were pure TSB and MH.

For testing the influence of pH, media whose pH values were 5.5, 6.5, 7, 7.5, 8.5, were prepared. After adding HCl (Zorka Pharma, Šabac, Serbia), acidic and neutral media (pH 5.5, 6.5, 7) were obtained, and after adding NaOH (Zorka Pharma, Šabac, Serbia), the basic media were obtained (pH 7.5, 8.5). All samples were prepared for each tested temperature (4°C, 37°C, 44°C). The samples were incubated for 24 h. For TSB growth control was on pH 7.5, while MH growth control was on pH 7.

For examining various salt concentrations, a modified TSB and MH broths with 4, 6.5 and 8% of NaCl were prepared (Zorka Pharma, Šabac, Serbia). All samples were

prepared for each tested temperature (4°C, 37°C, 44°C). The samples were incubated for 24 h. For determining the influence of different salt concentrations, we used TSB with 4% NaCl (the original, unmodified composition of the broth) and pure MH (MH did not contain any salt) which both served as controls. The quantity of salt added to the cheese was 6–8% of the cheese weight, since we wanted to examine how this salt concentration acted as a natural food preservative.

The bacterial growth in the tested broths, temperature, various pH values and different salt concentrations was measured spectrophotometrically at 600 nm. Measured higher absorbance means bacterial growth. Each experiment was performed in triplicate.

The determination of antibiofilm activity

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 which was described in Vestby et al. (2009), with modifications. TSB and MH media of 1.8 ml were inoculated with 0.2 ml of initial bacterial suspension (108 –109 CFU/ml) and then incubated for 96 h at 37°C. The 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.

Biofilm formation assay and quantification

The method described by O'Toole and Kolter (1998), with some modifications, was used for determining the ability of tested *Klebsiella* spp. to form biofilms. In sterile 96-well tissue culture plates (Sarstedt, Germany), which contained 100 µl of broth per well (TSB or MH) with different pH or various concentrations of salt, 10 µl of fresh bacterial suspension (108-109 CFU/ml) were added. 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 and crystal violet was added. Excess stain was rinsed off by thorough washing with deionized water and plates were fixed with 200 µl of 96% ethanol. Optical densities (ODs) of stained adherent bacteria were determined with a micro-ELISA plate reader (RT-2100C, Rayto, Shenzhen, China) at wavelength of 630 nm (OD630 nm).

The effect on formed biofilm

The effect of different pH and various salt concentrations on the formed biofilm of tested *Klebsiella* sp. was done as described in Mladenović et al. (2016a). 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 the incubation, the content of each well was gently pulled out. Next, 100 µl TSB or MH broth with different pH and salt concentrations was added to each well, and the microtiter plates were incubated at 37°C for 24 hours. After the incubation, the content of each well was gently removed by tapping the microtiter plates, and crystal violet was added. Excess stain was rinsed off by thorough washing with deionized water and plates were fixed with 200 µl of 96% ethanol. Optical densities (ODs) of stained adherent bacteria were determined with a micro-ELISA plate reader (RT-2100C, Rayto, Shenzhen, China) at wavelength of 630 nm (OD630 nm).

Data analysis

All data for planktonic and biofilm growth were presented as means ± standard deviations using Microsoft Excel (Redmond, Washington, DC, USA). A paired T-test was used for statistical processing of the results via IBM SPSS Statistics 20.

TABLE 1: *The effect of different pH on planktonic growth of bacteria at different temperatures.*

Broth	TSB[1]					MH[1]				
Species / pH	5.5	6.5	7	7.5*	8.5	5.5	6.5	7*	7.5	8.5
K. oxytoca KGPMF 2	0.02 ± 0.00	1.72 ± 0.03	1.66 ± 0.00	1.67 ± 0.00	1.61 ± 0.00	0.07 ± 0.02	0.50 ± 0.03	0.58 ± 0.05	0.61 ± 0.00	0.42 ± 0.04
K. oxytoca KGPMF 4	0.02 ± 0.00	0.62 ± 0.04	1.77 ± 0.02	1.64 ± 0.00	1.62 ± 0.00	0.06 ± 0.00	0.49 ± 0.08	0.48 ± 0.01	0.60 ± 0.01	0.20 ± 0.01
K. ornithinolytica KGPMF 9	0.02 ± 0.00	1.80 ± 0.02	1.72 ± 0.00	1.77 ± 0.01	1.73 ± 0.01	0.05 ± 0.00	0.62 ± 0.02	0.68 ± 0.00	0.62 ± 0.00	0.45 ± 0.00
K. pneumoniae KGPMF 11	0.03 ± 0.00	0.98 ± 0.02	1.05 ± 0.24	1.21 ± 0.22	1.16 ± 0.14	0.04 ± 0.00	0.67 ± 0.01	0.71 ± 0.01	0.75 ± 0.00	0.27 ± 0.02
K. pneumoniae ATCC 70063	0.03 ± 0.00	1.58 ± 0.25	1.74 ± 0.32	1.69 ± 0.16	1.73 ± 0.13	0.02 ± 0.00	1.28 ± 0.01	1.28 ± 0.04	1.20 ± 0.02	1.26 ± 0.08
Broth	**TSB[2]**					**MH[2]**				
Species / pH	5.5	6.5	7	7.5*	8.5	5.5	6.5	7*	7.5	8.5
K. oxytoca KGPMF 2	0.03 ± 0.00	0.81 ± 0.12	0.85 ± 0.1	0.84 ± 0.02	0.75 ± 0.08	0.02 ± 0.00	0.18 ± 0.01	0.16 ± 0.00	0.16 ± 0.03	0.06 ± 0.02
K. oxytoca KGPMF 4	0.04 ± 0.00	0.69 ± 0.03	0.72 ± 0.04	0.77 ± 0.03	0.85 ± 0.00	0.03 ± 0.00	0.19 ± 0.01	0.17 ± 0.04	0.19 ± 0.01	0.13 ± 0.00
K. ornithinolytica KGPMF 9	0.03 ± 0.00	0.59 ± 0.01	0.69 ± 0.02	0.73 ± 0.02	0.65 ± 0.00	0.10 ± 0.02	0.12 ± 0.00	0.10 ± 0.01	0.09 ± 0.00	0.09 ± 0.00
K. pneumoniae KGPMF 11	0.01 ± 0.00	0.12 ± 0.01	0.19 ± 0.00	0.28 ± 0.01	0.37 ± 0.03	0.04 ± 0.00	0.11 ± 0.00	0.12 ± 0.00	0.12 ± 0.01	0.12 ± 0.00
K. pneumoniae ATCC 70063	0.02 ± 0.00	0.68 ± 0.03	0.73 ± 0.04	0.62 ± 0.01	0.78 ± 0.02	0.04 ± 0.00	0.80 ± 0.00	0.69 ± 0.01	0.81 ± 0.07	0.35 ± 0.00

Values are presented as mean ± standard deviation measured by spectrophotometry at 600 nm;
*[1]Growth at 37°C; [2]Growth at 44°C; *Growth control*

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Results

The effect of different temperatures, pH and NaCl concentrations on bacterial planktonic growth in different growth media

Tested bacteria were grown in two different media at three temperatures (4°C, 37°C, and 44°C) in different pH. After the incubation, it was noticed that no growth occurred at 4°C. According to the measured absorbance, all tested bacteria demonstrated better growth in TSB than in MH, at 37°C. Higher absorbance exhibited better growth of the species. The limiting factor for the growth in TSB and MH was pH 5.5, at 37°C and 44°C. It can be concluded that growth at 44°C was lower than growth at 37°C in both broths. The results are presented in Table 1.

Tested bacteria were grown in two different media at three temperatures (4°C, 37°C, and 44°C) in different concentrations of NaCl. Different NaCl concentrations in TSB and MH at 37°C and 44°C demonstrated the reducing effect on the growth of all tested bacteria. Higher concentrations of salt exhibited the reducing effect on the growth of bacteria. Salt was a stronger limiting factor in MH than in TSB. The results are presented in Table 2.

TABLE 2: *The effect of different concentration of NaCl on planktonic growth of bacteria at different temperatures.*

Species	TSB[1]			MH[1]		
	4% *	6.5%	8%	4%	6.5%	8%
***K. oxytoca* KGPMF 2**	1.23 ± 0.03	0.56 ± 0.03	0.13 ± 0.01	0.23 ± 0.00	0.09 ± 0.00	0.03 ± 0.00
***K. oxytoca* KGPMF 4**	1.59 ± 0.01	0.86 ± 0.28	0.36 ± 0.04	0.11 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
***K. ornithinolytica* KGPMF 9**	1.42 ± 0.01	0.75 ± 0.02	0.25 ± 0.02	0.24 ± 0.00	0.09 ± 0.01	0.03 ± 0.00
***K. pneumoniae* KGPMF 11**	0.74 ± 0.02	0.60 ± 0.01	0.31 ± 0.00	0.13 ± 0.00	0.07 ± 0.00	0.02 ± 0.00
***K. pneumoniae* ATCC 70063**	1.69 ± 0.15	0.88 ± 0.05	0.28 ± 0.02	0.45 ± 0.00	0.13 ± 0.02	0.04 ± 0.00
Species	**TSB[2]**			**MH[2]**		
	4% *	6.5%	8%	4%	6.5%	8%
***K. oxytoca* KGPMF 2**	0.63 ± 0.03	0.35 ± 0.05	0.03 ± 0.01	0.06 ± 0.00	0.05 ± 0.04	0.02 ± 0.00
***K. oxytoca* KGPMF 4**	0.59 ± 0.02	0.44 ± 0.00	0.05 ± 0.02	0.06 ± 0.00	0.05 ± 0.00	0.01 ± 0.00
***K. ornithinolytica* KGPMF 9**	0.63 ± 0.00	0.46 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.00	0.01 ± 0.00
***K. pneumoniae* KGPMF 11**	0.61 ± 0.02	0.32 ± 0.03	0.17 ± 0.00	0.11 ± 0.03	0.05 ± 0.00	n.g.*
***K. pneumoniae* ATCC 70063**	0.54 ± 0.06	0.44 ± 0.00	0.04 ± 0.01	0.13 ± 0.08	0.06 ± 0.00	0.02 ± 0.00

*Values are presented as mean ± standard deviation measured by spectrophotometry at 600 nm; [1]Growth at 37°C; [2]Growth at 44°C; *Growth control*

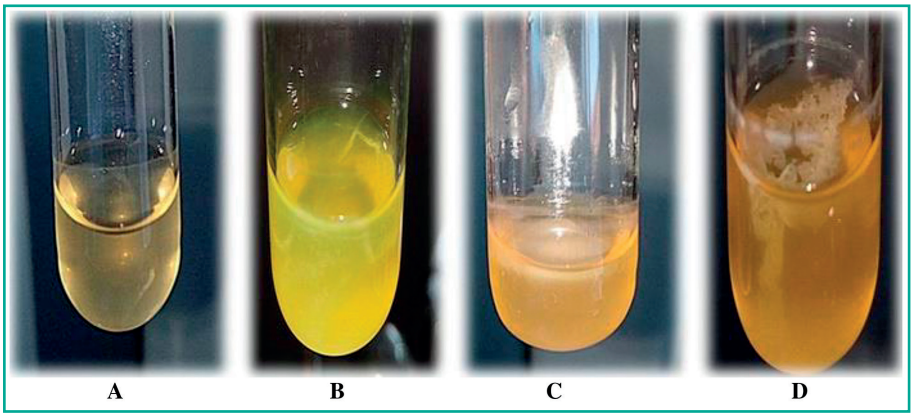

FIGURE 1: ***A:** Fromed pellicle of K. pneumoniae KGPMF 11 in MH; **B:** K. pneumoniae KGPMF 11 in TSB; **C:** K. pneumoniae ATCC 70063 in MH; **D:** K. pneumoniae ATCC 70063 in TSB.*

Pellicle test

After the incubation of 96 h, it was concluded that only *K. pneumoniae* KGPMF 11 and *K. pneumoniae* ATCC 70063 form pellicle. *K. pneumoniae* KGPMF 11 formed a very thin pellicle (weak biofilm producer) in MH, while in TSB they formed a solid fat pellicle (good biofilm producer). *K. pneumoniae* ATCC 70063, formed a thin pellicle (moderate biofilm producer) in MH, however, in TSB they formed solid (fat) pellicle (strong biofilm producer). It can be concluded that TSB was more suitable for the growth and development of the isolates, as well as for the biofilm formation. Pellicle producers are presented in Figure 1.

The effects of different temperatures and pH on biofilm formation and formed biofilm in TSB and MH

All bacteria were tested on the ability to form a biofilm at 4°C, 37°C, and 44°C. The results demonstrated that all tested bacteria, except *K. oxytoca* KGPMF 4 possessed the ability to form biofilm at 37°C. At 4°C and 44°C, tested bacteria demonstrated no ability to form a biofilm.

In all tested pH in TSB, the ability to form biofilm was reduced. The exception was *K. oxytoca* KGPMF 2, whose biofilm formation was stimulated in pH 5.5 (Figure 2). All tested pH demonstrated a stimulating effect on the formed biofilm in TSB of *K. pneumoniae* ATCC 70063, while pH 5.5 and 6.5 were stimulating formed biofilm of *K. oxytoca* KGPMF 2. All pH exhibited a reducing effect on the formed biofilm *K. pneumoniae* KGPMF 11 and *K. ornithinolytica* KGPMF 9 compared to the control. The results are presented in Figure 2.

All tested pH demonstrated a stimulating effect on the biofilm formation in MH for *K. oxytoca* KGPMF 2, except 8.5. For *K. ornithinolytica* KGPMF 9, pH 5.5 and 7.5 had a stimulating effect on the biofilm formation. All tested pH reduced biofilm formation of *K. pneumoniae* KGPMF 11 and *K. pneumoniae* ATCC 70063 (Figure 3). All pH exhibited a reducing effect on the formed biofilm of *K. oxytoca* KGPMF 2, except 7 and 7.5. Only pH 6.5 had a stimulating effect on formed biofilm of *K. ornithinolytica* KGPMF 9. All tested pH reduced a formed biofilm of *K. pneumoniae* KGPMF 11. Only pH 5.5 reduced a formed biofilm *K. pneumoniae* ATCC 70063. The results are presented in Figure 3.

The effects of different temperatures and NaCl concentrations on biofilm formation and formed biofilm in TSB and MH at 37°C

The influence of different salt concentrations on biofilm formation and formed biofilm of tested isolates, was investigated for the first time. 6.5% and 8% NaCl in TSB demonstrated reduced ability to form biofilm, except *K. pneumoniae* KGPMF 11 which showed no such ability in 8% NaCl. The results are presented in Figure 4. Tested salt concentrations demonstrated reducing effect on formed biofilm in TSB of tested bacteria. The exception was the formed biofilm of *K. pneumoniae* ATCC 70063, whose growth was stimulated under the influence of salt (Figure 4). In 4% NaCl and 6.5 % NaCl in MH, the ability to form biofilm of all bacteria was reduced, while in 8% NaCl, we did not observe the above-mentioned ability. It could be concluded that different salt concentrations demonstrated a reducing effect on formed biofilm. The results are presented in Figure 5.

Discussion

The effect of different temperatures, pH, salt concentrations and different growth media on the planktonic growth of *Klebsiella* spp. isolated from Sokobanja cheese (Southeastern Serbia) were investigated for the first time in this paper. The cheese belonged to the acid-curd soft cheese group (Mladenovic et al., 2018b; Mladenovic et al., 2018c), therefore, we wanted to examine how low pH affected enterobacteria. Moreover, we wanted to examine the impact of basic and neutral pH, in order to cover broader spectrum of ecological conditions. According to the results and comparing the two broths, it could be concluded that bacteria demonstrated better growth in TSB, since it was richer than MH due to its composition. The results of our study confirmed the planktonic growth of species of the genera *Klebsiella* at 37°C and 44°C in pH 5.5 and pH 8.5. Tsuji et al. (1982) investigated the growth of E. coli, *K. pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* at 25°C, 30°C, 37°C, and 42°C. They concluded that they grew equally well at all tested temperatures and that they were destroyed after 30 min at 60°C or 70°C. Our research proved that the temperature of 37°C was more suitable for planktonic growth than 44°C, however, this difference was not statistically significant. According to Brisse et al. (2006), *K. ornithinolytica* grew at 5°C, while *K. pneumoniae* and *K. oxytoca* did not grow. Our research indicated that *K. ornithinolytica*, *K. pneumoniae* and *K. oxytoca* isolated from cheese demonstrated no growth at 4°C, after 24 h. This was due to the fact that although they belonged to the same genus, the strains might exhibit different growth characteristics. Furthermore, our research confirmed the ability of *K. pneumoniae* to grow at 44°C. The effect of TSB on the planktonic growth and biofilm formation was statistically significant, compared to the effect on the planktonic growth and biofilm formation in MH ($p < 0.05$). The effect of both tested

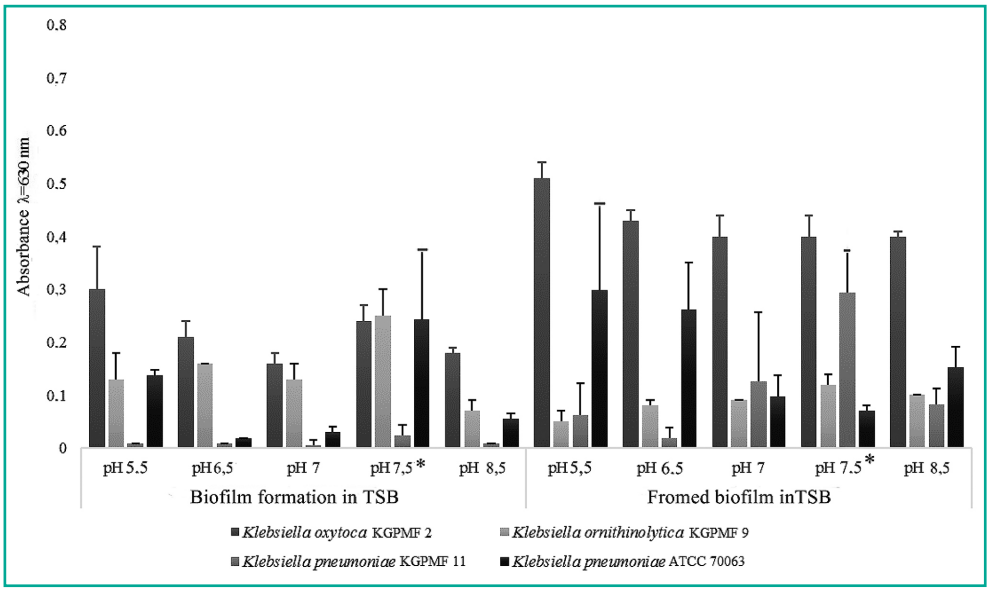

FIGURE 2: *Effect of different pH concentration on biofilm formation and formed biofilm in TSB at 37 °C (*growth controls).*

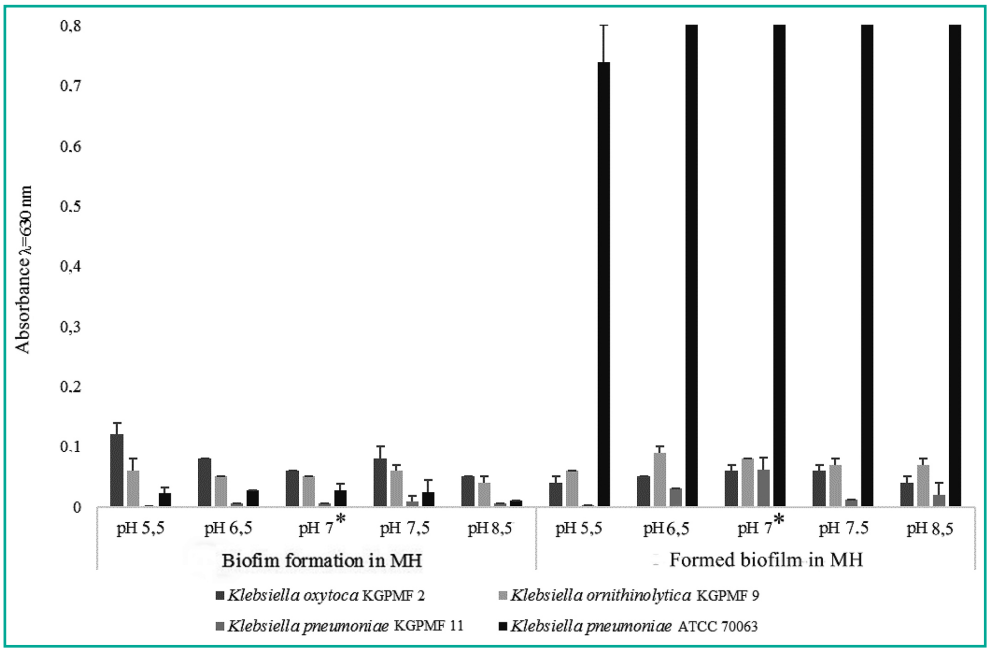

FIGURE 3: *Effect of different pH concentration on biofilm formation and formed biofilm in MH at 37 °C (*growth controls).*

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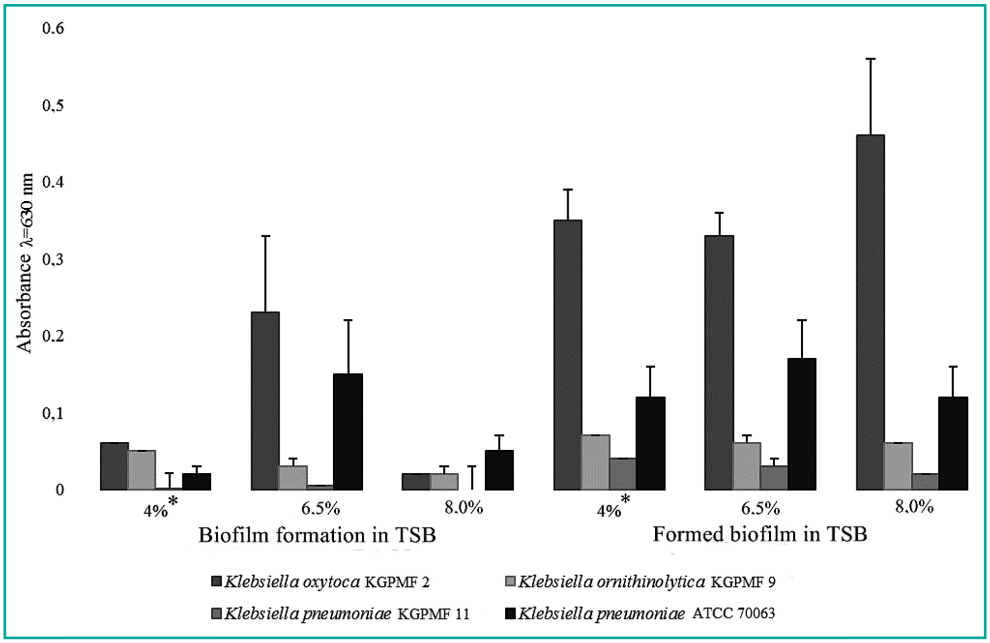

FIGURE 4: *Effects of different salt concentration on biofilm formation and formed biofilm in TSB at 37 °C (*growth controls).*

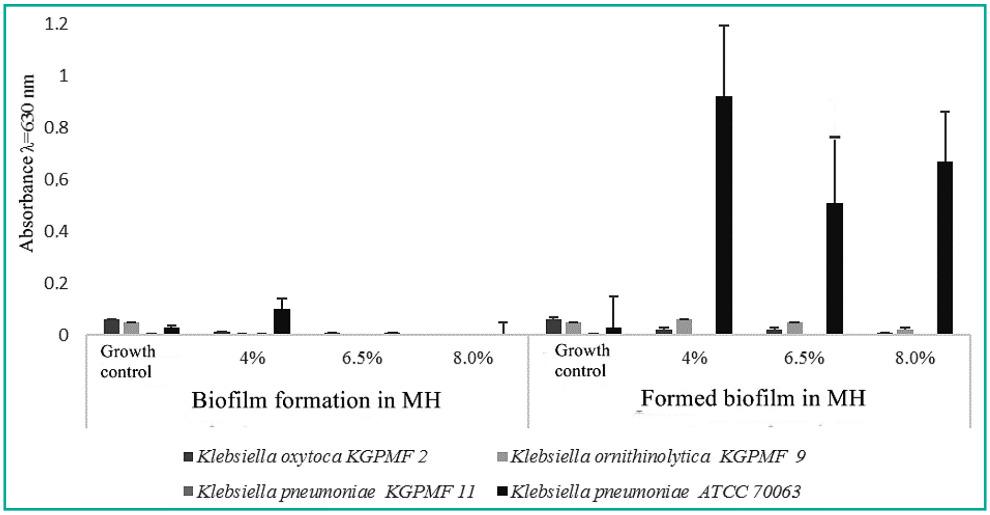

FIGURE 5: *Effects of different salt concentration on biofilm formation and formed biofilm in MH at 37 °C.*

broths on formed biofilm was not statistically significant ($p > 0.05$). Heretofore, the influence of various concentrations of glucose and lactose on the planktonic growth of the same isolates of *Klebsiella* spp. was investigated. The results indicated that sugars produced stimulating effects on their growth (Mladenović et al., 2018d). Any change in the environment may favor the development of biofilm. Bacteria use the ability to form biofilm as an adaptation as well as the stress response to environmental changes. Therefore, the examination of various environmental factors is an important factor in the examination of biofilms, especially those of natural strains.

In this study, the ability of bacteria isolated from cheese to form biofilm and the influence of pH and salt on the formation and formed biofilm were investigated for the first time. TSB is more suitable for the biofilm of strains than MH. This is also observed in pellicle formation. It was noticed that the pellicle was stronger in TSB than in MH of *K. pneumoniae* 11 and ATCC strain at 37 °C (Figure 1). The biofilm of *K. pneumoniae* was already investigated since it was concluded that formed biofilm possessed resistance to antibiotics (Ribeiro et al., 2016). *K. pneumoniae* which was isolated from sputum and surgical-wound swabs produced fully established biofilms, which was different from other isolates (Seifi et al., 2016). The ATCC 70063 strain was used as standard in our research. The ATCC strain established characteristics, without mutations. *K. pneumoniae* from cheese was different from the ATCC strains. Due to the fact that strains found in the environment were adapted well to specific conditions, they demonstrated modifications compared to ATCC strains. Environmental isolates, in our case those of cheese, may be more adaptable than the standard strain. They can adapt faster and more easily to different variations of environmental factors. The results of our study indicated that bacteria from the genus *Klebsiella* were also sensitive to low temperature, acidic and alkaline environment, and high salt concentration. At 37°C, bacterial growth was statistically higher in MH broth, at pH 8.5 ($p = 0.03$). In other combination of broths and pH, the growth was higher at 37°C, but the difference was not statistically significant ($p>0.05$) Therefore, it could be concluded that pH had a higher influence on bacterial growth, than temperature, in both tested broths, at both tested temperatures. Regarding the salt concentrations, temperature of 37°C created statistically significant impact on bacterial growth in TSB with 4% of salt ($p = 0.0005$) and in MH broth with 4% of salt ($p = 0.01$) and 6.5% of salt ($p = 0.002$). Bacterial growth was low at 8% of salt in both broths, at both tested temperatures. Based on the results, it could be concluded that tested bacteria were adapted to grow in salt concentrations up to 6.5% and that temperature was a more limiting factor than salt, in these cases. Moreover, the influence of sugars on the biofilm formation of *Klebsiella* species isolated from the same cheese was investigated (Mladenovic et al., 2018d). Other scientists have also reported similar results to ours. *Klebsiella* spp. isolated from the meat demonstrated the ability to form biofilm (Manges, 2015). According to Hoštacká et al. (2010), the *K. pneumoniae* biofilm formation increased in pH 8.5. According to the research conducted by Mirkar et al. (2016), a high salt concentration of 8% and above, low temperature and alkaline pH (pH 9) may prevent the biofilm formation by *K. pneumoniae*.

In addition to different values of pH and salt, concentrations of sugar also affect the biofilm growth of strains. The biofilm formation was less affected by glucose. However, lactose stimulated biofilm formation (Mladenović et al., 2018d).

Previous investigation of members of fam. Enterobacteriaceae isolated from Sokobanja cheese showed that temperature of 4°C, low pH, and all concentrations of salt exhibited an inhibitory effect on the planktonic growth of *Serratia* species in MH ant TSB media (Mladenović et al., 2018e). The acidic medium and salt were limiting factors for the growth of *Escherichia* species, while the basic medium was more suitable for the growth. Biofilm formation of *Serratia* and *Escherichia* isolates was possible only at 37°C. Serratia and Escherichia isolates showed different abilities to form biofilm in acidic and alkaline mediums. Generally speaking, the strongest biofilm formation was at pH 7 and 7.5. Different salt concentrations demonstrated the reducing effect on biofilm formation of *Serratia* and *Escherichia* isolates (Mladenović et al., 2018c; Mladenović et al., 2018e).

Conclusion

The different processing conditions (temperature, amount of salt, pH, etc.) are substantial for the planktonic growth and biofilm formation of enterobacteria. The bacteria from the genera *Klebsiella* isolated from Sokobanja cheese, demonstrated sensitivity to acidic and alkaline pH by decreasing the growth, demonstrated lower growth at 44°C, and did not grow at 4°C, which was important since the cheese was stored in a refrigerator. Salt may be used as a preservative since a higher concentration of it produced an inhibitory effect on planktonic growth and biofilm formation, as well as on the formed biofilm. Further investigations are required in order to include the influence of other processing conditions.

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Conflict of Interest

The authors declare no conflict of interest.

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