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The simbiotic effect of temperature and sugars on the planktonic growth and biofilm formation of *Klebsiella* spp. isolated from traditionally made cheese

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Abstract

In this paper, we investigated the influence of temperatures (4°C, 37°C, 44°C) and different concentrations of glucose and lactose (0.5%, 1.5%, 2.5%, 3.5%) on the planktonic growth, biofilm formation and formed biofilm of Klebsiella oxytoca, Klebsiella ornithinolytica and Klebsiella pneumonia in two different broths (TSB and MH). The bacteria were isolated from the cheese produced in Southeastern Serbia. Klebsiella pneumoniae ATCC 70063 was used as a positive control. The planktonic growth and biofilm formation were measured using spectrophotometric method. Different broths caused a different planktonic growth and biofilm formation. At 4°C, the planktonic growth was not observed, neither was the formation of biofilm. Temperatures of 37°C and 44°C, as well as various concentrations of glucose and lactose, stimulated the planktonic growth of bacteria. The biofilm formation was less affected by glucose, but lactose stimulated the biofilm formation. The results of these were in accordance with the origin of bacteria, since the isolates were obtained from cheese. The intensity of the effect of sugars on the planktonic growth and biofilm formation depended on the type of bacteria.

Keywords: *Klebsiella*, planktonic growth, biofilm, temperature, glucose, lactose

1. Introduction

Fam. Enterobacteriaceae is a common constituent of dairy products microbiota. Contamination of dairy products may occur during milking process, production, storage or transportation (V. ZARATE & al. [1]; M. HATZIKAMARI & al. [2]; E. NIKOLAOU & al.

[3]). Cheese is an excellent source of protein, fat, minerals (calcium, iron), vitamins and amino acids (U. GUVEN & al. [4]). Certain concentrations of sugars, glucose, fructose, and lactose are present in these products (M. KHANGHOLI & al. [5]). The bacteria of the genera *Klebsiella* are often found in a variety of environmental conditions, such as soil, vegetation, and water, including the drinking water distribution system (W.R. JARVIS & al. [6]; R. PODSCHUN & al. [7]; R. PODSCHUN & al. [8]). *Klebsiella* spp. was isolated from local Nigerian cheese (A. SANGOYOMI & al. [9]).

The parameters which have the effect on the growth of microorganisms in food products are: pH, moisture content, nutrient content, antimicrobial constituents, etc. (J. JAY, [10]). The external sources of nitrogen, energy (carbohydrates, proteins and fats), minerals and vitamins are necessary for the development of microorganisms (M. ŠKRINJAR *&* al. [11]). When pathogenic bacteria leave their hosts, they are often challenged by various environmental stresses, such as nutrient starvation, osmotic shock, temperature variation, oxidative stress in natural environments (O. BRENHORVD & al. [12]; S.N. BOUCHER & al. [13]; M.L. HÄNNINEN & al. [14]). Bacteria utilize various methods for protection from assorted environmental conditions. One of the many methods involves biofilm formation, but this formation depends on many factors. The type and concentration of substances such as sugars, which are present in the environment around bacteria, might be among effective facilitators of the biofilm formation (M. KHANGHOLI & al. [5]). The biofilm formation was stronger if the low concentration of glucose (0.5% or 1.5%) was used (J.C.M. COSTA & al. [15]).

The aim of this study was to investigate the planktonic growth and biofilm formation of *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Klebsiella ornithinolytica* in two different broths, under the influence of various temperatures and different concentrations of glucose and lactose. Another objective of this study was to examine the influence of the mentioned environmental factors on formed biofilm.

2. Materials and Methods

Strains and growth conditions

The bacteria used in this study were: *Klebsiella oxytoca* KGPMF 2, *Klebsiella oxytoca* KGPMF 4, *Klebsiella ornithinolytica* KGPMF 9, *Klebsiella pneumoniae* KGPMF 11. The bacteria were previously isolated from Serbian cheese (Sokobanja region) and determined at

the Laboratory for Microbiology at the Faculty of Science, University of Kragujevac (KGPMF) (K. MLADENOVIĆ & al. [16]). The collection of identified bacterial species was kept in a 20% glycerol/medium mixture at -80°C. As a positive control, *K. pneumonia* ATCC 70063 was used.

The analysis of the effect of temperature and different concentrations of glucose and lactose on the planktonic growth

The effect of different temperatures on the planktonic growth of *Klebsiella* spp. was investigated in TSB and Muller-Hinton broth. These two broths were used due to the fact they differed in composition and had different influence on the bacterial growth. The effect of different concentrations of glucose and lactose (0.5%, 1.5%, 2.5%, 3.5%, respectively) in modified TSB and MH broth was investigated. In 3 mL of each type of media, we added 10 μL of initial bacterial suspension (10^8-10^9 (CFU).mL^{-1}). All samples were prepared in triplicate, each for one tested temperature (4°C, 37°C, 44°C) and incubated for 24 h. Growth in TSB containing 0.25% of glucose and pure MH served as a growth control. The results were obtained using spectrophotometer at 600 nm.

Determination of antibiofilm activity

The analysis of the biofilm formation and quantification

The ability of *K. oxytoca* KGPMF 2, *K. oxytoca* KGPMF 4, *K. pneumoniae* KGPMF 11, *K. ornithinolytica* KGPMF 9, *K. pneumoniae* ATCC 70063 to form biofilms was assayed as described (G. O'TOOLE & al. [17]) with some modifications.

For the experiment, two different broths (TSB and MH broth) were used. 100 μL of broth which contained different concentrations of glucose and lactose (0.5%, 1.5%, 2.5%, 3.5%, respectively) and 10 μL of fresh bacterial suspension (1.0 McFarland) were added in sterile 96-well tissue culture plates (Sarstedt, Germany). After the 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 wells were washed with 200 μL of sterile saline to remove free-floating bacteria. Biofilms formed by adherent cells in plate were fixed using 100 μL of methanol, then stained with 100 μL (0.1%) of crystal violet and incubated at the room temperature for 20 min. Crystal violet was washed three times with 200 μL of deionized water and then100 μL of 96% ethanol was added to each plate. Optical densities (ODs) of stained adherent bacteria were determined using the enzyme-linked immunosorbent assay (ELISA) plate reader (RT-2100C, Rayto, Shenzhen, China) at 630 nm.

A sterile broth containing different concentrations of sugar served as a control to check sterility and nonspecific binding of media. To compensate for background absorbance, OD

readings from sterile medium, modified broth, fixative, and dye were averaged and subtracted from all test values. All tests were performed in triplicate and their mean value was calculated.

The effect on formed biofilm

The 96-well tissue culture microtiter plates (Sarstedt, Germany) were prepared by dispensing 100 μL of broth. 10 μL of fresh bacterial suspension (1.0 McFarland) was added into 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. Then, we added 100 μL of broth which contained various concentrations of glucose and lactose (0.5%, 1.5%, 2.5%, 3.5%, respectively) and inoculated 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. After that, the experiment was assayed likewise previously described modifications.

Data analysis

All data were presented as means ± standard deviations where it was appropriate, using Microsoft Excel (Redmond, Washington, DC, USA). Paired T-test was used for processing the results of the bacterial growth in two broths statistically, via IBM SPSS Statistics 20.

3. Results and discussion

The effect of different concentrations of glucose on the planktonic growth on bacteria

Tested bacteria were grown in different media (TSB and MH broth), enriched with different concentrations of glucose and lactose, at three temperatures (4°C, 37°C, 44°C). After the incubation, it was noticed that there was no growth at 4°C.

K. oxytoca KGPMF 2 in TSB containing 0.5% and 1.5% of glucose at 37°C, demonstrated higher growth, but growth in 2.5% was the same as the growth control. The growth of *K. oxytoca* KGPMF 4 and *K. pneumoniae* KGPMF 11 in all concentrations of glucose was lower. *K. ornithinolytica* KGPMF 9 in 0.5% and 2.5% of glucose showed higher growth. *K. pneumoniae* ATCC 70063 demonstrated higher growth in all tested concentrations.

In MH broth at 37°C, the growth of *K. oxytoca* KGPMF 2 and *K. ornithinolytica* KGPMF 9 in all concentrations of glucose was stimulated, while the growth of *K. oxytoca* KGPMF 4 was similar to the growth control in 0.5% and 1.5% of glucose, except for the stimulated growth in 2.5%. The growth of *K. pneumoniae* KGPMF 11 was lower in all tested concentrations. *K pneumoniae* ATCC 70063 showed higher growth in all concentrations, except in the concentration of 3.5% (Table 1).

The growth at the temperature of 44°C, in both broths was lower than at 37°C. *K. oxytoca* KGPMF 2, *K. oxytoca* KGPMF 4 and *K. pneumoniae* KGPMF 11 in TSB with 0.5% and 1.5% glucose demonstrated stimulated growth. The growth of *K. ornithinolytica* KGPMF 9 in 0.5% was the same as the growth control. *K. pneumoniae* ATCC 70063 in 0.5% and 1.5% showed the same growth as the growth control, while in 3.5% growth was higher.

In MH broth, *K. oxytoca* KGPMF 2, *K. oxytoca* KGPMF 4, *K. ornithinolytica* KGPMF 9, *K. pneumoniae* KGPMF 11, in all concentrations demonstrated stimulated growth. *K. pneumoniae* ATCC 70063 showed stimulated growth in 1.5% and 3.5% of glucose (Table 1).

Table 1. The effect of different concentrations of glucose on the planktonic growth of bacteria at 37°C and 44°C

Species	TSB with glucose at 37°C					TSB with glucose at 44°C				
	0.5%	1.5%	2.5%	3.5%	Growth control	0.5%	1.5%	2.5%	3.5%	Growth control
K. oxytoca KGPMF 2	1.92±0.01	2.0±0.02	1.65±0.01	1.49±0.00	1.67±0.00	0.97±0.03	0.93±0.08	0.83±0.09	0.80±0.15	0.84±0.02
K. oxytoca KGPMF4	1.25±0.01	1.16±0.08	1.05±0.03	1.01±0.00	1.64±0.00	0.89±0.01	0.87±0.02	0.75±0.05	0.70±0.07	0.77±0.03
K. ornithinolytica KGPMF9	2.03±0.00	1.73±0.01	2.00±0.02	1.59±0.04	1.77±0.01	0.74±0.04	0.69±0.03	0.61±0.03	0.54±0.11	0.73±0.02
K. pneumoniae KGPMF11	0.87±0.00	0.77±0.01	0.68±0.01	0.65±0.01	1.21±0.22	0.34±0.00	0.30±0.00	0.26±0.00	0.20±0.02	0.28±0.01
K. pneumoniae ATCC 70063	2.19±0.12	2.07±0.08	1.88±0.06	1.89±0.06	1.69±0.16	0.69±0.01	0.61±0.02	0.50±0.02	0.86±0.03	0.62±0.01
Species	MH broth with glucose at 37°C					MH broth with glucose at 44°C				
	0.5%	1.5%	2.5%	3.5%	Growth control	0.5%	1.5%	2.5%	3.5%	Growth control
K. oxytoca KGPMF2	1.15±0.04	1.11±0.03	1.04±0.17	1.32±0.29	0.58±0.05	0.33±0.08	0.37±0.05	0.49±0.04	0.34±0.05	0.16±0.00
K. oxytoca KGPMF4	0.43±0.02	0.41±0.03	0.74±0.02	0.34±0.02	0.48±0.01	0.39±0.00	0.41±0.02	0.40±0.01	0.37±0.00	0.17±0.04
K. ornithinolytica KGPMF 9	1.19±0.02	0.94±0.00	1.06±0.03	0.82±0.00	0.68±0.00	0.26±0.01	0.23±0.00	0.31±0.00	0.18±0.00	0.10±0.01
K. pneumoniae KGPMF11	0.43±0.00	0.39±0.01	0.44±0.00	0.31±0.00	0.71±0.01	0.29±0.02	0.27±0.01	0.26±0.00	0.23±0.01	0.12±0.00
K. pneumoniae ATCC 70063	1.49±0.01	1.30±0.03	1.75±0.07	1.11±0.03	1.28±0.04	0.63±0.05	1.10±0.05	0.31±0.01	1.58±0.17	0.69±0.01

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

In the TSB and MH broth containing lactose, *K. oxytoca* KGPMF 2, *K. ornithinolytica* KGPMF 9 and *K. pneumoniae* ATCC 70063 showed stimulated growth while *K. oxytoca* KGPMF 4 and *K. pneumoniae* KGPMF 11 demonstrated lower growth at 37°C (Table 2).

The growth at 44°C was lower in all tested concentrations of lactose in both media than the growth at 37°C. In TSB, *K. oxytoca* KGPMF 2, *K. oxytoca* KGPMF 4, *K. ornithinolytica* KGPMF 9 showed stimulated growth in TSB in all concentrations of lactose, except in 3.5%. *K. pneumoniae* KGPMF 11 and *K. pneumoniae* ATCC 70063 in all tested concentrations

demonstrated higher growth. In MH broth, all tested bacteria showed higher or the same growth compered to growth control (Table 2).

Table 2. The effect of different concentrations of lactose on the planktonic growth on bacteria at 37°C and 44°C

Species	TSB with lactose at 37♌°C					TSB with lactose at 44°C				
	0.5%	1.5%	2.5%	3.5%	Growth control	0.5%	1.5%	2.5%	3.5%	Growth control
K. oxytoca KGPMF 2	2.01±0.02	1.94±0.03	2.05±0.02	1.95±0.02	1.67±0.00	0.99±0.06	0.97±0.07	0.89±0.02	0.82±0.05	0.84±0.02
K. oxytoca KGPMF4	1.51±0.01	1.20±0.02	1.17±0.01	1.09±0.01	1.64±0.00	1.01±0.02	0.89±0.04	0.84±0.02	0.74±0.02	0.77±0.03
K. ornithinolytica KGPMF9	2.09±0.02	2.10±0.04	2.13±0.03	2.06±0.02	1.77±0.01	0.75±0.02	0.73±0.03	0.77±0.02	0.68±0.04	0.73±0.02
K. pneumoniae KGPMF11	1.19±0.00	0.84±0.01	0.70±0.01	0.64±0.03	1.21±0.22	0.37±0.03	0.39±0.02	0.39±0.01	0.34±0.02	0.28±0.01
K. pneumoniae ATCC 70063	2.03±0.11	2.16±0.09	2.24±0.09	2.05±0.11	1.69±0.16	0.68±0.04	0.65±0.02	0.68±0.01	0.65±0.00	0.62±0.01
Species	MH broth with lactose at 37°C					MH broth with lactose at 44°C				
	0.5%	1.5%	2.5%	3.5%	Growth control	0.5%	1.5%	2.5%	3.5%	Growth control
K. oxytoca KGPMF2	1.19±0.02	1.17±0.02	1.13±0.01	1.08±0.00	0.58±0.05	0.44±0.02	0.46±0.01	0.41±0.04	0.41±0.05	0.16±0.00
K. oxytoca KGPMF4	0.48±0.02	0.51±0.04	0.44±0.01	0.43±0.03	0.48±0.01	0.50±0.12	0.55±0.00	0.50±0.00	0.49±0.00	0.17±0.04
K. ornithinolytica KGPMF 9	1.12±.0.02	1.04±0.01	0.95±0.02	0.86±0.01	0.68±0.00	0.50±0.02	0.55±0.06	0.50±0.06	0.49±0.02	0.10±0.01
K. pneumoniae KGPMF11	0.59±0.02	0.49±0.02	0.48±0.03	0.47±0.03	0.71±0.01	0.29±0.00	0.27±0.00	0.22±0.06	0.26±0.01	0.12±0.00
K. pneumoniae ATCC 70063	1.56±0.02	1.46±0.02	1.48±0.06	1.49±0.10	1.28±0.04	1.52±0.02	1.62±0.13	1.70±0.09	1.69±0.04	0.69±0.01

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

In this paper, the effect of various temperatures and different concentrations of glucose and lactose on the planktonic growth of bacteria from cheese produced in Southeastern Serbia were investigated for the first time. The effect of glucose on the planktonic growth at 37°C and 44°C depends on the type of bacteria, but growth was lower at 44°C in both broths. Lactose stimulated the growth of bacteria in both broths at both temperatures, which was anticipated, since the bacteria were isolated from cheese. The results of this study confirmed that the effect of sugar depended on the type of bacteria. The same conclusion was given by (M. KHANGHOLI & al. [5]).

The effects of different concentrations of glucose on the biofilm formation and formed biofilm in TSB and MH broth

All bacteria were tested for their ability to form biofilm at the 4°C, 37°C and 44°C. The results showed that all tested bacteria possessed the ability to form biofilm only at 37°C, except *K. oxytoca* KGPMF 4.

Biofilm formation

TSB containing 0.5% and 2.5% of glucose stimulated the biofilm formation of *K. oxytoca* KGPMF 2, while other concentrations reduced the biofilm formation. All tested concentrations of glucose caused lower ability to form biofilm of *K. ornithinolytica* KGPMF 9, *K. pneumoniae* KGPMF 11 and *K. pneumoniae* ATCC 70063, except the concentration of 3.5%, which caused increased biofilm formation of *K. ornithinolytica* KGPMF 9.

Under the influence of 0.5%, 1.5%, 3.5% of glucose in MH broth, the biofilm formation of *K. oxytoca* KGPMF 2 was stimulated. The concentrations of 0.5% and 1.5% of glucose reduced biofilm formation of *K. ornithinolytica* KGPMF 9, while other tested concentrations stimulated the biofilm formation. The influence of 0.5% of glucose, caused the reduced biofilm formation of *K. pneumoniae* KGPMF 11, while other concentrations stimulated the formation of biofilm. All tested concentrations of glucose stimulated biofilm formation of *K. pneumoniae* ATCC 70063 (Figure 1**)**.

Formed biofilm

The influence of TSB containing different concentrations of glucose showed stimulating effect on the formed biofilm of *K. oxytoca* KGPMF 2 and *K. pneumoniae* KGPMF 11, except for the concentration of 0.5% which caused reduction of the formed biofilm. The influence of 0.5% and 1.5% of TSB containing glucose stimulated the growth of the formed biofilm of *K. ornithinolytica* KGPMF 9, while other concentrations reduced the formed biofilm. All concentrations of glucose stimulated the growth of formed biofilm *K. pneumoniae* ATCC 70063, compared to the growth control.

MH broth containing different concentrations of glucose showed stimulating effect on the formed biofilm of *K. oxytoca* KGPMF 2, *K. ornithinolytica* KGPMF 9 and *K. pneumoniae* ATCC 70063. 0.5% and 1.5% of glucose stimulated the formed biofilm of *K. pneumoniae* KGPMF 11 (Figure 1).

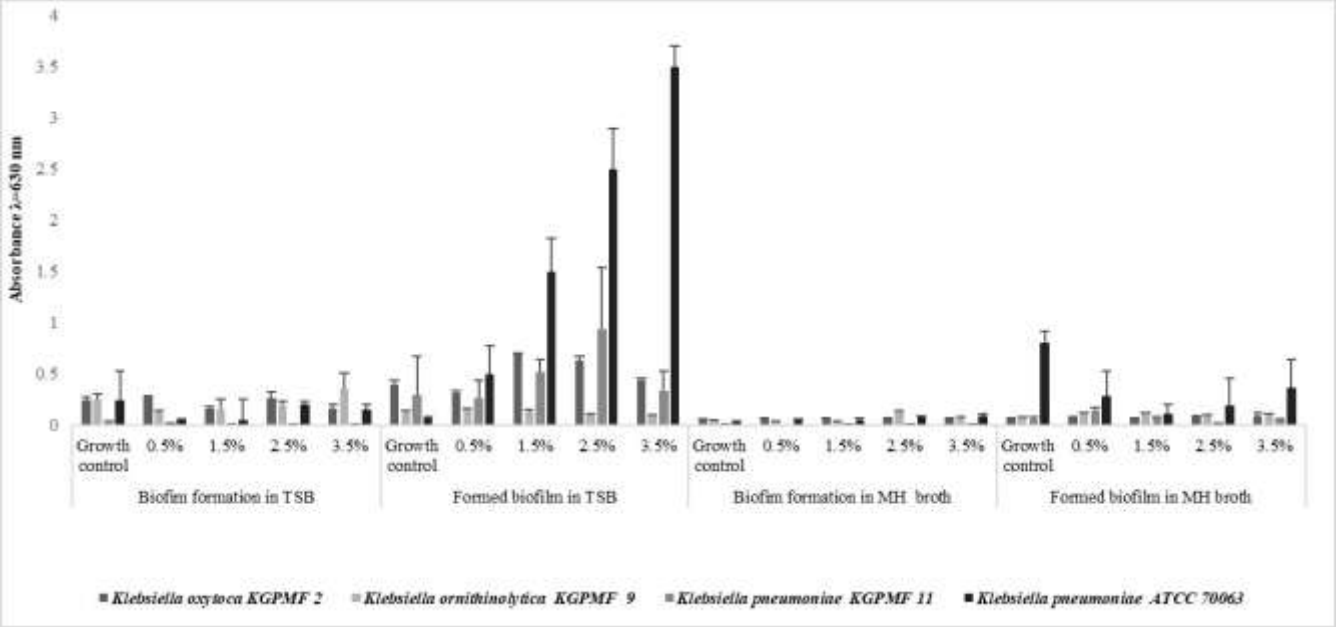

Figure 1. The effects of different concentrations of glucose on the biofilm formation and formed biofilm in TSB and MH broth

The effects of different concentrations of lactose on the biofilm formation and formed biofilm in TSB and MH broth

Biofilm formation

TSB containing different concentrations of lactose caused stronger biofilm formation of *K. oxytoca* KGPMF 2. *K. ornithinolytica* KGPMF 9, *K. pneumoniae* 11 KGPMF and *K. pneumoniae* ATCC 70063 formed weaker biofilm, except *K. pneumoniae* KGPMF 11 which in the concentrations of 0.5% and 3.5% of lactose, formed the same biofilm as the growth control.

MH broth containing different concentrations of lactose, showed the ability to reduce biofilm formation of *K. oxytoca* KGPMF 2 and *K. pneumoniae* KGPMF 11, except in 0.5% of lactose, where the biofilm formation of *K. pneumoniae* KGPMF 11 was the same as the growth control. All concentrations of lactose showed stimulating effect on the biofilm formation of *K. ornithinolytica* KGPMF 9, except in the concentration of 3.5% where biofilm formation was weaker. All concentrations of lactose stimulated the biofilm formation of *K. pneumoniae* ATCC 70063 (Figure 2**)**.

Formed biofilm

TSB containing different concentrations of lactose demonstrated stimulating influence on the formed biofilm of *K. oxytoca* KGPMF 2 and *K. pneumoniae* ATCC 70063. *K. ornithinolytica*

KGPMF 9 in 0.5% of lactose caused stimulated growth of the formed biofilm, while other concentrations showed reducing effect on the formed biofilm. All concentrations of lactose showed reducing effect on formed biofilm of *K. ornithinolytica* KGPMF 11.

MH broth containing different concentrations of lactose demonstrated stimulating effect on the formed biofilm of *K. oxytoca* KGPMF 2 and *K. pneumoniae* ATCC 70063. The concentrations of 0.5% and 1.5% of lactose had stimulating effect on the formed biofilm of *K. ornithinolytica* KGPMF 9. 2.5% of lactose demonstrated stimulating effect on the formed biofilm of *K. pneumoniae* KGPMF 11. The effect of different concentrations of lactose on the formed biofilm, was stronger compared to the effect they had on the biofilm formation (Figure 2).

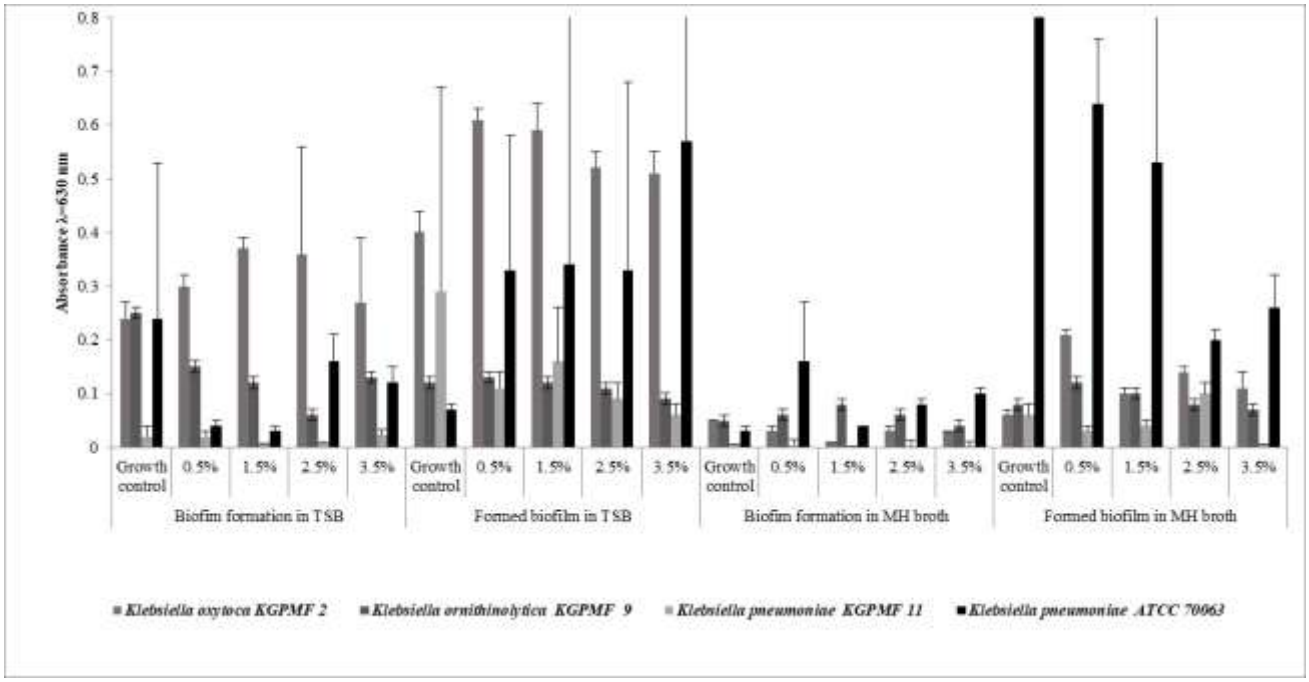

Figure 2. The effects of different concentrations of lactose on the biofilm formation and formed biofilm in TSB and MH broth

The planktonic growth and the biofilm formation in TSB were statistically significant at both tested temperatures, compared to the planktonic growth and biofilm formation in MH broth at the same temperature ($P < 0.05$). The effect of broths on the formed biofilm was not statistically significant ($P > 0.05$).

In this paper, we examined for the first time the effect of different temperatures and concentrations of glucose and lactose on the biofilm formation and formed biofilm of bacteria from the cheese made in Southeastern Serbia. Different concentrations of lactose

demonstrated stronger effect on the biofilm formation and formed biofilm, compared to different concentrations of glucose.

Changes in the environment, may cause changes in the bacterial cell and in biofilm formation (J.W. COSTERTON & al. [18]); T.K. JANA & al. [19]). According to (M. KHANGHOLI & al. [5]) sugar concentration reduces the level of bacteria and biofilm formation in yoghurt. In our research, different concentrations of glucose showed a stimulating or inhibitory effects, depending on the type of bacteria. According to (D. W. JACKSON & al. [20]) sugars can inhibit the formation of biofilm. In our research, all tested concentrations of glucose, stimulated the growth of formed biofilm of *K. pneumoniae* ATCC 70063.

It is possible that the sugar level and temperature produce a synergistic effect on the biofilm formation (Y. PAN & al. [21]). Our research showed that temperature and some glucose and lactose concentrations demonstrated a synergistic effect and stimulated the growth of bacteria. If we compare all the factors tested in this paper, which may affect the planktonic growth, biofilm formation and formed biofilm, it can be concluded that the temperature of 4°C inhibited the planktonic growth and biofilm formation. Different concentrations of glucose and lactose in TSB and MH broth at 37°C and 44°C, produced a stimulating or inhibitory effect, depending on the type of bacteria.

4. Conclusion

The results of this study contribute to better understanding of unexplored microflora of cheese from Southeastern Serbia. Environmental conditions are very important for the planktonic growth of bacteria and biofilm formation. The bacteria of the genus *Klebsiella* show susceptibility only at a low temperature (4°C). Cheese contains a lot of lactose, numerous substrates for growth, but the temperature is crucial for the metabolic activity of bacteria. In a further study, a synergistic effect of various ecological factors on the metabolic activity of bacteria from cheese made in Southeastern Serbia, should be investigated.

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