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In vitro evaluation of resistance to environmental stress by planktonic and biofilm form of lactic acid bacteria isolated from traditionally made cheese from Serbia

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ABSTRACT

In this study, the effects of different temperature, pH, different concentrations of salt, glucose and lactose, on the planktonic growth, biofilm formation and formed biofilm of *Enterococcus hirae* KGPMF9, *Streptococcus uberis* KGPMF2, *Lactococcus lactis* subsp. *lactis* KGPMF23 and *Lactobacillus fermentum* KGPMF29 was investigated. These lactic acid bacteria were previously isolated from traditionally made cheese from Southeastern Serbia. Evaluation of the effect of different environmental conditions on the planktonic growth, biofilm formation and formed biofilm were determined by spectrophotometric method. The limiting factor for the planktonic growth of tested bacteria was the salt concentration above 6.5%, while temperature of 4 °C was limiting factor for planktonic growth and biofilm formation. Temperature of 37 °C as well as various concentrations of glucose and lactose, stimulated planktonic growth and biofilm formation of all tested bacteria, except *E. hirae* KGPMF9. *S. uberis* KGPMF2 showed no ability of biofilm formation. Tested bacteria showed better planktonic growth and ability of biofilm formation in acidic media. Basic media was limiting factor for biofim formation. These results provide a basis for further research of influence of more environmental conditions on the development of lactic acid bacteria and their use like probiotics or starter cultures.

1. Introduction

Lactic acid bacteria (LAB) are widely used in food fermentation and it plays an important role in the development of the organoleptique and hygienic quality of fermented products. But, in the fermentation process, they need to survive in variable environmental conditions including differences in temperature, different type of sugar, pH and salinity (Rao, Pintado, Stevens, & Guyot, 2004). Also, these environmental conditions play significant role in adhesion and biofilm formation of bacteria (Mirkar, Rawat, & Satish, 2016). Gravesen, Lekkas, and Knochel (2005) suggested that the biofilm formation is bacterial stress response on environmental conditions. Abdallah, Benoliel, Drider, Dhulster, and Chihib (2014) also has suggested that environmental conditions, which can be met in food and medical area, also enhance the biofilm formation. Van de Guchte, Serror, Chervaux, Smokvina, and Ehrlichet (2002) indicated that LAB evolved defense mechanisms against stress that allow them to survive in harsh conditions and sudden environmental changes.

According to the International Union of Pure and Applied Chemistry, biofilm is an "aggregate of microorganisms in which cells that are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface" (Vert et al., 2012). It is well-known that bacteria from milk have the ability to form multispecies biofilms (Teh et al., 2014). Some studies have reported the ability of LAB isolated from cheese to form biofilm (Gómez, Ramiro, Quecan, & de Melo Franco, 2016; Somers, Johnson, & Wong, 2001). Winkelströter, Reis, Silva, Alves, and De Martins (2013) indicated that biofilms formed by LAB present in foods may offer a promising means to counteract the establishment of pathogenic biofilms. Crowley, Leigh, Ward, Lappin-Scott, and Bowler (2011) has concluded that biofilm formation of *S. uberis* is correlated with an up regulation of several gene products, which are important for pathogenesis. Macovei et al. (2009) indicated that *E. hirae* produce an unknown weak protease that does not contribute to biofilm formation. Lactobacilli that showed the ability of biofilm formation were able to control development of *Listeria monocytogenes* on abiotic surfaces (Pérez-Ibarreche, Castellano, & Vignolo, 2014). Salas-Jara, Ilabaca, Vega, and García (2016) investigated and verified the beneficial effect of *Lactobacillus fermentum* in biofilm form, including increased resistance to temperature and gastric pH. Kubota, Senda, Tokuda,

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Uchiyama, and Nomura (2009) investigated the stress responses of biofilm and planktonic cells of *Lb. plantarum* subsp. *plantarum*. They concluded that planktonic bacterial cells showed reduced resistance to acetic acid, but the bacterial cells in biofilms maintained their resistance. Also, they suggested the importance of controlling biofilms of LAB in the food industry. Oxaran et al. (2012) has demonstrated the capacity of *Lactococcus lactis* to form biofilms on solid surfaces. Gaglio et al. (2016) investigated the ability of *Lc. lactis* subsp. *cremoris* strains to develop biofilms on the surfaces of virgin wooden vats, which were used for cheese production. They showed that, in the presence of *Lc. lactis* subsp. *cremoris* biofilm on the surfaces of virgin wooden vats and addition of the natural whey starter culture, the microbial variability in cheese was reduced.

Dairy products could be contaminated with some bacteria, which presence is undesired (Koch et al., 2010). Some of undesired bacteria, especially L. *monocytogenes,* have the ability to survive for a long time exposure to diverse adverse conditions, including acidic pH, low temperatures, and high sodium chloride concentrations. This ability made this kind of organism difficult for control in food (Farber & Peterkin, 1991). On the other hand, LAB, isolated from dairy products, showed the ability to produce bacteriocins, which showed the ability to prevent the growth and development of undesired bacteria in dairy products (Scatassa et al., 2015, 2017). Some studies have reported the use of bacteriocin produced by LAB for biopreservation of cheeses (Hernández, Cardell, & Zárate, 2005; O'Sullivan, O'Connor, Ross, & Hill, 2006). But, it has been reported that, some process conditions, such as different pH, culture media and temperature can affect to bacteriocin production of some *Lactobacillus* and *Lactococcus* species (Aasen, Møretrø, Katla, Axelsson, & Storrø, 2000; Juarez Tomás, Bru, Wiese, De Ruiz Holgado, & Nader-Macías, 2002; Mataragas, Metaxopoulos, Galiotou, & Drosinos, 2003).

Therefore, the aim of this study was the *in vitro* evaluation of the influence of environmental conditions (different temperature, pH, and different concentration of sodium chloride, glucose and lactose) on the planktonic growth and ability of biofilm formation by LAB. Also, the aim was the evaluation of the influence of the mentioned environmental factors on the formed biofilm.

2. Materials and methods

2.1. Test microorganisms

The effects of pH, sodium chloride (NaCl), glucose, lactose and temperature on the planktonic growth, biofilm formation and formed biofilm were tested against 4 species of bacteria isolated from cheese from Southeastern Serbia: *Enterococcus hirae* KGPMF9, *Streptococcus uberis* KGPMF2, *Lactococcus lactis* subsp. *lactis* KGPMF23 and *Lactobacillus fermentum* KGPMF29 (Muruzović, Mladenović, Zugić Petrović, & Comić, 2018). All isolates from cheese were provided by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The bacterial strains were kept in glycerol stock at − 80 °C.

2.2. Assay for determination of the effect of different temperature, pH, different concentration of sodium chloride, glucose and lactose on planktonic growth of bacteria

Assay for determination of the effect of different environmental conditions on planktonic growth of bacteria was performed according to Thayer, Muller, Buchanan, and Phillips (1987), with some modifications described below. Planktonic cells of tested bacteria were incubated statically in glass tubes containing 5 ml of MRS broth (Torlak, Belgrade, Serbia) under aerobic conditions for 24 h at 37 °C. Initial bacterial suspensions contained 10^8 colony-forming units CFU/ml. Planktonic growth was measured at spectrophotometer at 600 nm. Absorbance values of sterility controls were subtracted from the absorbance values of the tested samples, in order to recompense the background absorbance. Each experiment was performed in triplicate.

2.2.1. Effect of different temperature

The effect of temperature on the growth of tested bacteria was examined in standard or modified MRS broth. In 3 ml of MRS broth, it was added 10 µl of initial bacterial suspension. All samples were prepared in triplicate, each for one tested temperature (4 °C, 20 °C and 37 °C). Samples were incubated for 24 h. Sterility control was pure MRS broth.

2.2.2. Effect of different pH

Modified MRS broths, whose pH values were 5.5, 6.5, 7, 7.5, and 8.5, were prepared for examination of the effect of pH. Growth control was at pH 6.5. With adding HCl (Zorka Sabac, Sabac, Serbia), it was obtained acidic media and with adding NaOH (Zorka Sabac, Sabac, Serbia), it was obtained neutral and basic media of MRS. In 3 ml of each type of modified media, it was added 10 µl of initial bacterial suspension. Samples were incubated at 4 °C, 20 °C and 37 °C for 24 h. The results were determined with spectrophotometer at 600 nm. Each experiment was performed in triplicate.

2.2.3. Effect of different concentration of sodium chloride

MRS broths, modified with the addition of NaCl (4%, 6.5%, 8%) were prepared for examination of different effect of NaCl. In 3 ml of modified media, it was added 10 µl of initial bacterial suspension. Samples were incubated at 4 °C, 20 °C and 37 °C for 24 h. The results were determined with spectrophotometer at 600 nm. Each experiment was performed in triplicate.

2.2.4. Effect of different concentration of glucose and lactose

Modify MRS broths, with different concentrations of glucose and lactose (Torlak, Belgrade, Serbia) (0.5%, 1.5%, 2.5%, 3.5%, respectively) were prepared for the examination. In 3 ml of modified media, it was added 10 µl of initial bacterial suspension. Samples were incubated at 4 °C, 20 °C and 37 °C for 24 h. The results were determined with spectrophotometer at 600 nm. Each experiment was performed in triplicate.

2.3. Determination of antibiofilm activity

2.3.1. Biofilm formation assay and quantification

The ability of tested bacteria to form biofilms, as well as the influence of different environmental conditions to the ability of bacteria to form biofilms, was assayed as described by O'Toole and Kolter (1998) and Stepanovic et al. (2007), with some modifications.

The tissue culture 96-well microtiter plates (Sarstedt, Germany) were prepared by dispensing 100 µl of MRS broth, which contained different pH (5.5, 6.5, 7, 7.5, 8.5), different percent of NaCl (4%, 6.5%, 8%), glucose and lactose (0.5%, 1.5%, 2.5%, 3.5%, respectively), separately, in each well. The rest of the experiment was done as described in Muruzović, Mladenović, Stefanović, Vasić, and Čomić (2016).

2.3.2. Effect on formed biofilm

The influence of different environmental conditions on the already formed biofilm of tested bacteria, was evaluated. The tissue culture 96-well microtiter plates (Sarstedt, Germany) were prepared by dispensing 100 µl of MRS broth. 10µl of fresh bacterial suspension (1.0 McFarland) was added into each well. The inoculated microtiter plates were incubated at 37 °C for 24 h. After incubation, the content of each well was gently pulled out. Then, it was added 100 µl of MRS broth, which contained different pH (5.5, 6.5, 7, 7.5, 8.5), different percent of NaCl (4%, 6.5%, 8%), glucose and lactose (0.5%, 1.5%, 2.5%, 3.5%, respectively) separately, and inoculated microtiter plates were incubated at 37 °C for 24 h. After incubation, the content of each well was gently removed by tapping the microtiter plates. The rest of the experiment was done as described in Muruzović et al. (2016).

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