PERIODICUM BIOLOGORUM
VOL. 116, No 1, 89–93, 2014

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

# Influence of a medium pH and ionic strength on the adhesion of *Streptococcus thermophilus* microorganisms to human erythrocytes

**MARIANA ANIKIEIEVA**[1]
**OLGA GORDIYENKO**[2]

[1] V.N. Karazin Kharkiv National University,
4 Svoboda Sq., Kharkiv, 61022, Ukraine

[2] Institute for Problems of Cryobiology
and Cryomedicine NAS of Ukraine,
23 Pereyaslavskaya str., 61015, Kharkov, Ukraine

**Correspondence:**
Mariana Anikieieva
V.N. Karazin Kharkiv National University,
Svoboda Sq., Kharkiv, 61022, Ukraine
E-mail: maryano4ka2008@ukr.net

Received February 26, 2014.

## Abstract

***Background and Purpose:*** *Diseases caused by bacterial pathogens are the result of the host-pathogen interaction, therefore the contact of bacteria with erythrocytes is highly possible. The aim of this work was to study* Streptococcus thermophilus *adhesion on human erythrocytes depending on the various pH and ionic strength of the medium.*

***Materials and Methods:*** Streptococcus thermophilus *bacterial cells and human red blood cells have been selected. Erythrocytes were washed with buffer saline and precipitated by centrifugation. Dried bacterial cells were suspended, incubated, washed in buffer saline and precipitated. The precipitate of both types of cells was resuspended in 1:2 ratio in a buffer solution with different physicochemical characteristics. The number of bacteria adhered to each erythrocyte has been counted under light microscope in each field of view and adhesion coefficient has been calculated.*

***Results:*** *Adhesion coefficient is strongly dependent on pH of the medium and reaches maximum at physiological pH for red blood cells. Data for the other examined pH values probabilistically differ at the same confidence level. Adhesion index was the highest in physiological saline (2.21 ± 0.87). Both increase or decrease in the ionic strength (0.2 M salt) resulted in the drop of adhesion rate, it becomes minimal (0.95 ± 0.63) in the medium with the lowest investigated ionic strength (0.25 M sucrose, 0.025 M salt).*

***Conclusions:*** *The results of our experiments indicate that adhesion proceeds through initial nonspecific stage which plays an important role in the adhesion process and affects the possibility of the second irreversible stage of adhesion.*

## INTRODUCTION

Bacterial pathogens interact with host cells resulting in infectious deseases. Infection proceeds through colonization or penetration into deep host tissues where bacteria synthesize specialized protein factors, allowing them to bind to host cells. Adhesin receptors usually represent hydrocarbons of surface exposed glycoproteins or glycolipids, often covalently attached to membrane proteins of epithelial cells in the target tissue or organ. These hydrocarbons are often found in the same glycocompounds, proteins or sialoglycoproteins of cell membranes in other places except the target tissue or organ. For instance, red blood

cells exhibit a huge variety of complex glycoproteins, glycosphingolipids and gangliosides which are identical or similar to adhesin receptors on epithelial cells. Thus, red blood cells are a convenient source of mammalian cells, which have a large number of exposed complex carbohydrates, which are potentially related carbohydrate sequences for bacterial adhesins. For example, adhesion of hemagglutinin of *Helicobacter pilori* to the blood cell membrane has characteristics similar to those observed when bacteria attach to epithelial cells *(1)*.

On the other hand, germs of bacterial nature that get into the bloodstream interact with various components of plasma and blood cells. The most likely is a contact of bacteria with erythrocytes. However, there is almost no information on the role of germ adhesion to erythrocytes, which largely determines the development of the immune response and infectious processes. Mechanisms of bacterial adhesion to the surface of red blood cells had been poorly studied. That is why a more detailed study of the influence of the temperature and environmental parameters on bacterium fixing activity to erythrocytes is of importance.

In this work we investigated the dependence of *Streptococcus thermophilus* adhesion to human red blood cells on pH and ionic strength of the medium.

## MATERIALS AND METHODS

The ability of lactobacilli chain adhesion to human erythrocytes has been monitored. We used *Streptococcus thermophilus* bacterial cells as a model.

Red blood cells were washed twice with physiological 0.1 M phosphate buffer saline at pH 7.4 and precipitated by centrifugation at 2,000 rpm for 5 minutes. The dried bacterial cells were suspended in saline supplemented with 5% glucose and incubated at 37 °C for 30 minutes. Then, they were washed in saline and collected by centrifugation at 6,000 rpm once more. The precipitates of both types of cells were resuspended in 1:2 ratio in buffer solution (specify which solution) with appropriate physicochemical characteristics.

Cell suspension has been mixed with an experimental solution in 1:1 ratio and incubated at 37°C for 30 minutes, shaking every 5 minutes. Adhesion of bacterial cells to human erythrocytes was examined by means of Axio Observer Z1 microscope (oil-immersion lens ×63). To calculate the number of adhered bacteria, five different fields of view (before and after each mechanical shake of the sample) have been recorded. Within each field of view the number of adhered bacteria to each erythrocyte has been counted and the average number of adhered bacteria to a single erythrocyte - adhesion coefficient - has been calculated.

To examine the influence of the medium pH the precipitates of both cell types were resuspended in 1:2 ratio in buffered saline, pH 5.8, 6.6, 7.4 and 8.0.

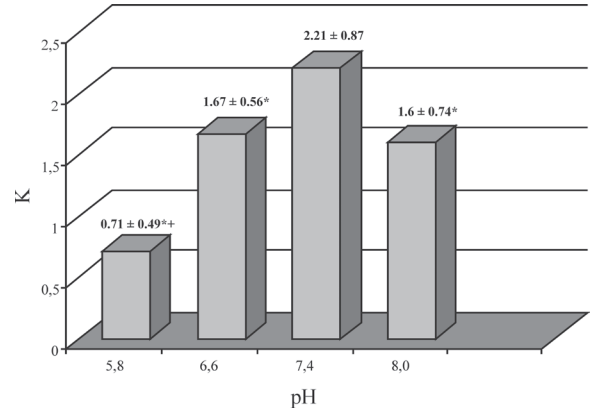


**Figure 1.** *The dependence of adhesion coefficient K of bacteria cells* Streptococcus thermophilus *to human erythrocytes on medium H.*
*Note: * – data differ significantly from data for pH 7.4; + – data likely differ from data for pH 6.6 and 8.0; p < 0.001.*

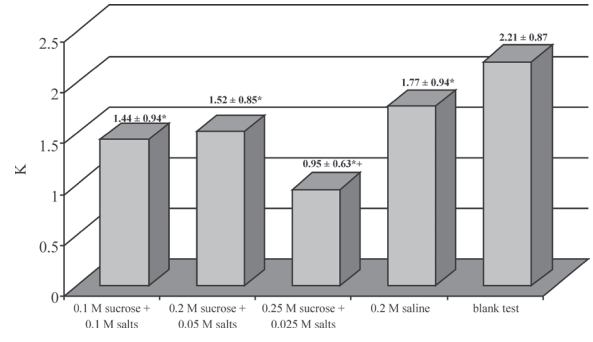


**Figure 2.** *The dependence of adhesion coefficient K of bacterial cells* Streptococcus thermophilus *to human erythrocytes on the ionic strength of the medium.*
*Note: * – data differ significantly from data for blank test; p < 0.001; + – data differ significantly from data for any other examined media.*

In experiments assessing the influence of ionic strength of the medium on the adhesion coefficient of bacterial cells to erythrocytes the medium ionic strength has been reduced by replacing electrolytes with sucrose to maintain the solution osmolality at a physiological level. The following solutions have been used in the experiment: N1: 0.1 M sucrose + 0.1 M salts; N2: 0.2 M sucrose + 0.05 M salts; N3: 0.25 M sucrose + 0.025 M salts; N4: 0.2 M saline; blank test: a physiological buffered solution. All the solutions were at pH 7.4.

## RESULTS

The influence of the medium pH on the process of *Streptococcus thermophilus* adhesion has been examined. Figure 1 shows the adhesion coefficients obtained for different pH values and the adhesion coefficients obtained for solutions of different ionic strength at pH 7.4 are presented in Figure 2.

Results show, that the adhesion coefficient is strongly dependent on the pH of the medium and has maximum at physiological values for red blood cells. Adhesion coefficients for pH 5.8 (281 erythrocytes were counted), 6.6 (166 erythrocytes were counted), and 8.0 (91 erythrocytes were counted) differ significantly ($p< 0.001$) from those for pH 7.4 (283 erythrocytes were counted). Data for pH 5.8 and other examined pH values probabilistically differ at the same confidence level. However adhesion coefficients for pH 6.6 and 8.0 do not probabilistically differ.

At physiological pH values the adhesion coefficient is the largest in physiological saline. Increasing the salt concentration from to 0.2 M resulted in the adhesion coefficient decrease by 20 % (however the difference is not probabilistic). Lowering the ionic strength of the medium leads to a decrease in adhesion. The adhesion coefficient was shown to be the lowest in the medium of the minimal ionic strength (solution N3). The value of adhesion coefficient in this medium probabilistically differs from those for all other investigated media. The following number of erythrocytes were counted in the examined solution: 0.1 M sucrose + 0.1 M salts - 124 erythrocytes, 0.2 M sucrose + 0.05 M salts - 122 erythrocytes, 0.25 M sucrose + 0.025 M salts - 199 erythrocytes, 0.2 M saline - 134 erythrocytes, blank test - 283 erythrocytes.

## DISCUSSION

Adhesion is a complex process controlled by many special systems. The process of bacterial adhesion is usually discussed in terms of the two-stage sorption model which has been proposed by Marshall and others *(2)*. According to this model, at the first stage bacteria quickly attach to the surface by weak physical interactions, forming mostly reversible attachment, while at the second irreversible stage molecular and cellular adhesion processes take place, and the aggregates resistant to any washing processing are formed *(3)*. Therefore, after some time, the adhesive bond between the bacteria and the substrate surface becomes stronger, transforming the process into irreversible state. Microbial desorption has been studied in situ in the device with controlled flow as a function of retention time of organisms on the surface. We have found that *S. thermophilus* desorption significantly reduces within about 50 seconds after the initial adhesion due to strengthening of the bonds. Previously atomic-force microscopy (AFM) was used to confirm the strengthening of the bonds between the cell surface of *S.thermophilus* and silicon nitride tips. These data were consistent with the macroscopic data obtained for microorganisms desorption under the action of flow, depending on the exposure time. AFM has shown the strengthening of bonds between the tip and the cell surface within 100 seconds of exposure, i.e. within the time interval of the same order as the time necessary for the strengthening of the bonds depending on the exposure time during desorption. The authors believe that the comparison of the interaction energy observed by means of AFM and macroscopic desorption method indicates that the bond's strength increases as a result of multi-attachments of extracellular polymeric substances to the substrate surface *(4)*. However, strengthening of the bonds with time has been also found in experiments with quartz fibers *(5)*. Effect of the slow destruction of boundary layers, which were losing their stability due to mutual overlap, was observed. As a result, adhesion strength increases with increasing contact time.

In our experiments, bacteria have been incubated with erythrocytes for 30 minutes. Thus, the data obtained reflect the result of the final (irreversible) stage. However, the impact of the first stage of attachment certainly affects the final result. From physico-chemical point of view, the initial instantaneous phase of microbial adhesion is mediated by nonspecific interactions with the characteristics of long-range action, including Lifshitz – van der Waals forces, electrostatic forces, acid-base and hydrophobic interactions and the forces of Brownian motion. Depending on the relatively limited number of physical and chemical properties, it is usually considered within Derjaguin-Landau-Verwey-Overbeek (DLVO) theory *(5-7)*. Bacterial adhesion to many materials has been successfully described in terms of colloidal interaction forces due to physico-chemical properties of bacteria and surfaces *(6)*. Bacteria can have multiple adhesins for different substrates, usually lectins or lectin like proteins or carbohydrates, parts of the surface polymeric structures that include capsules, villi or piles. However, many studies have claimed that these structural features are less important in the initial stages of binding than thermodynamic factors *(8, 9)*, and a number of detailed studies have been undertaken to support this assertion *(10, 11)*. Extended DLVO theory, proposed in *(5, 12)*, uses components of both models and includes acid-alkali interactions (hydrophobic/hydration effects) in addition to the classical van der Waals and electrostatic interactions. In some cases, the extended DLVO theory qualitatively predicts the experimental adhesive results better than the classical DLVO theory and thermodynamical approach separately.

Therefore, adhesion of eukaryotic cells as well as microorganisms is a complicated process, which is influenced by many factors, including some properties of the cells themselves (hydrophobicity, surface charge, specific adhesins, virulence factors, etc.), material surface of the object (chemical composition, inequality, wettability, etc.) and external factors (temperature, exposure time, number of cells, the presence of antibiotics, chemicals, etc.). pH of the medium can affect adhesion in several aspects. The total surface charge is determined by the components of the cell wall. Because of different pH values, the surface chemical groups, such as carboxyl, hydroxyl, and phosphate groups undergo protonation at different levels, which affects the surface charge *(13, 14)*. Most of lactobacilli lines usually have isoelectric point between 3 and 4.5 *(13, 15)*, and in some cases even below 2 *(14, 16)*.

In the case of two different cell surfaces studied here the situation is further complicated as the changes on both surfaces will affect the resulting adhesion. It was shown that the initial desorption rate coefficients for *S. thermophilus* binding to the glass surface, were higher at pH 2 than that at pH 7, i.e. the binding of *S.thermophilus* at the initial stage was stronger at pH 7 than in an acidic medium (pH 2). These results are consistent with the data obtained by atomic force-microscopy *(4)*.

The adhesion of BHK cells to various polymeric surfaces with measured densities of hydroxyl and carboxyl groups was studied in *(18)*. The impact of blocking of hydroxyl groups by acetylation and carboxyl groups by diazomethane on cell adhesion has been examine. It has been shown that hydroxyl groups are required for cell adhesion, however high surface density of these groups decreases cell adhesion, i.e. there is some optimum surface density of OH groups for BHK cells adhesion. Carboxyl groups inhibited cell adhesion to some extent. Observed increase in adhesion when blocking these groups by methylation can be taken as evidence of the process.

On the other hand pH of the medium can influence the properties of adhesins and receptors. For example, in *(19)* it was shown that purified adhesins isolated from *Bacteroides loeschei*, are basic proteins with pI values between 7.4 and 8.0. The authors believe that the adhesins recognize the same sugars in the prokaryotic and eukaryotic receptors, although the nature of these receptors is probabilistically very different. A bacterial receptor is probably similar to a polysaccharide of a cell wall, whereas an erythrocyte receptor is likely a glycoprotein or glycolipid. The properties of the purified adhesins were dependent on pH of the medium and its the ability to agglutinate red blood cells and streptococcus microorganisms were only developed in a neutral medium with pH 6.8 and hasn't developed in an acidic medium with pH 4.6.

In our case, the *S.thermophilus* adhesion to erythrocytes was also the highest at physiological pH values and decreased by changing the pH both to acidic or alkaline value.

Increase in the electrolyte concentration leads to either a decrease in electrostatic potential of the surface as a result of counterions adsorption, or compression of the diffuse ionic layer, or to both at the same time; in any case it is accompanied by the lowering of barrier repulsion. When a certain electrolyte concentration is reached the attractive forces become dominant at all distances *(5)*. For some microorganisms the influence of ionic strength of the suspending solution on hydrophobicity of a cell surface has been shown. Hydrophobicity of the cell surface of certain lines of lactobacilli was shown to change in response to alterations in ionic strength *(20)*. Two selected lines exhibited dynamic cell surface hydrophobicity - the line of lactobacilli with SLP (*L Lactobacillus acidophilus* ATCC 4356) was hydrophobic in 10 mM KCl and became more hydrophilic in 100 mM KCl, whereas the line without SLP (*Lactobacillus casei* ATCC 393) was hydrophilic in 10 mM KCl and became hydrophobic in 100 mM KCl. The authors believe that the dynamic behavior of bacterial cell surfaces, when ionic strength is changing, provides multilateral mechanism of adhesion to hydrophobic and hydrophilic surfaces in solutions with low and high ionic strength for certain lines of lactobacilli.

In *(21),* the role of cell surface hydrophobicity of two lactobacilli lines with and without surface layer of proteins (SLP-layer) was investigated with regard to their adhesion to hydrophobic or hydrophilic surfaces at low and high values of the ionic strength of the suspension. At low ionic strength of the suspension, both lactobacilli lines demonstrate higher initial rate of attachment to a glass hydrophobic surface than to a hydrophilic one, while at the high ionic strength of the suspension a clear influence of a cell surface hydrophobicity on adhesion was not observed. In *(22)* it was shown that the number of adhered *Streptococcus. oralis 34* microorganisms after 4 hours of incubation was higher in 50 mM KCl, than in 15 mM KCl, and decreased in 250 mM KCl. $\varsigma$-potential of investigated oral microorganisms decreased by absolute value from -20 mV in 2 mM KCl to -10 mV in 100 mM KCL. The main reduction of $\varsigma$-potential occurs up to the concentration of 60 mM. When the mentioned concentration is reached the curve of $\varsigma$-potential change reaches the plateau.

According to extended DLVO theory, at the initial stages of adhesion in addition to electrostatic and dispersion interactions, the so-called structural strengths are considered. They were discovered in experiments measuring the power threshold by crossed filaments. In solutions of low ionic strength the satisfactory agreement with theoretical estimates by DLVO theory is observed. However, in solutions with a concentration of more than 0.1 mol/l additional forces, not taken into account by the theory, appeared. These forces did not disappeared neither at the point of zero charge, nor with further increase in the electrolyte concentration. Structural forces appear between surfaces when the thickness of the aqueous layer between them h ≤ 70-80Å, i.e. when the effect of their sharp exponential increase with decreasing thickness start to contribute increasingly. Structural repulsion forces in the range of short distances undergo a dramatic exponential rise with the characteristic length in the order of 10Å. Thus for distances h <50Å the increase in concentration of 1-1 electrolyte to 0.01 mol/L had little effect on the structural forces, and at the higher concentration boundary aqueous layers begin to deteriorate rapidly. At a distance of h> 50Å differences in the drop of structural forces are also observed, they are more long-range in solutions of low ionic strength, i.e. increase in electrolyte concentration reduces the structural forces range and results in their sharp decrease *(5)*.

Thus, the results of our experiments, showing the dependence of adhesion of *S.thermophilus* microorganisms

to human erythrocytes on pH and ionic strength of the suspending solution, are consistent with the provisions of the extended DLVO theory. These results indicate that the first non-specific stage plays an important role in the process of adhesion and affects the possibility of the second irreversible stage of adhesion.

## REFERENCES


1. EVANS D G, EVANS D J 1995 Adhesion properties of *Helicobacter pilori*. Methods in Enzimology. Academic Press 253, p 336-360
2. HERMANSSON M 1999 The DLVO theory in microbial adhesion. *J. Colloids and Surfaces B: Biointerfaces 14:* 105–119
3. CARNAZZA S 2009 New Advances in Cell Adhesion Technology: Lecture Notes in Nanoscale Science and Technology 7. *In:* Nanoparticles and Nanodevices in Biological Applications, Bellucci *(ed.).* Springer-Verlag, Berlin, Heidelberg, p 69-130
4. VADILLO-RODRIGUEZ V, BUSSCHER H J, NORDE W, DE VRIES J, VAN DER MEI H C 2004 Atomic force microscopic corroboration of bond aging for adhesion of *Streptococcus thermophilus* to solid substrata. *Journal of Colloid and Interface Science 278*: 251–254
5. ISRAELACHVILI J 2011 Intermolecular and Surface forces. - 3rd ed. *In:* Academic Press, Burlington, p 674
6. BOS R, VAN DER MEI H C, BUSSCHER H J 1999 Physico-chemistry of initial microbial adhesive interactions – its mechanisms and methods for study. *FEMS Microbiol Rev 23*: 179–230
7. NEU T R, MARSHALL K C 1990 Bacterial polymers: Physicochemical aspects of their interactions at interfaces. *J Biomater Appl 5*: 107–133
8. AN Y H, FRIEDMAN R J 1998 Concise review of mechanisms of bacterial adhesion to biomaterial surfaces. *J Biomed Mat Re 43:* 338–348
9. MORRA M, CASSINELLI C 1997 Bacterial adhesion to polymer surfaces: a critical review of surface thermodynamic approaches. *J Biomater Sci Polymer Ed 9*: 55–74
10. CUNLIFFE D, SMART C A, ALEXANDR C, VULFSON E N 1999 Bacterial adhesion at synthetic surfaces. *Appl Environ Microbiol 65:* 4995–5002
11. VAN LOOSDRECHT M C M, LYKLEMAM J, NORDE W, ZEHNDER A J B 1990 Hydrophobic and electrostatic parameters in bacterial adhesion. *Aquatic Sci 52:* 103–113
12. VAN OSS C J 1989 Energetics of cell-cell and cell-biopolymer interactions. *Cell Biophys 14:* 1–16
13. SCHAR-ZAMMARETTI P, UBBINK J 2003 The cell wall of lactic acid bacteria: Surface constituents and macromolecular conformations. *Biophys J 85*: 4076–4092
14. DEEPIKA G, CHARALAMPOPOULOS D 2010 Surface and Adhesion Properties of Lactobacilli. *Advances in Applied Microbiology 70:* 127-152
15. PELLETIER C, BOULEY C, CAYUELA C, BOUTTIER S, BOURLIOUX P, BELLON-FONTAINE M N 1997 Cell surface characteristics of *Lactobacillus casei* subsp *casei*, *Lactobacillus paracasei* subsp *paracasei*, and *Lactobacillus rhamnosus* strains. *Appl Environ Microbiol 63:* 1725–1731
16. REID G, BIALKOWSKA-HOBRZANSKA H, VAM DER MEI H C, BUSSCHER H J 1999 Correlation between genetic, physicochemical surface characteristics and adhesion of four strains of Lactobacillus. *Colloid Surf 13*: 75–81
17. CURTIS A S G, FORRESTER J V, CLARK P 1986 Substrate hydroxilation and cell adhesion. *J Cell Sci. 86:* 9-24
18. LONDON J, ALLEN J 1990 Purification and Characterization of a *Bacteroides loeschei* Adhesin That Interacts with Procaryotic and Eucaryotic Cells. *J of bacteriology 5:* 2527-2534
19. VADILLO-RODRIGUEZ V, BUSSCHER H J, NORDE W, DE VRIES J, VAN DER MEI H C 2004 Dynamic Cell Surface Hydrophobicity of *Lactobacillus* Strains with and without Surface Layer Proteins. *J Bacteriology 186 (19):* 6647–6650
20. VADILLO-RODRIGUEZ V, BUSSCHER H J, VAN DER MEI H C, DE VRIES J, NORDE W 2005 Role of lactobacillus cell surface hydrophobicity as probed by AFM in adhesion to surfaces at low and high ionic strength. *Colloids and Surfaces B: Biointerfaces 41:* 33–41
21. BOS R, VAN DER MEI H C, H. BUSSCHER H J 1996 Influence of ionic strength and substratum hydrophobicity on the co-adhesion of oral microbial pairs. *Microbiology 142*: 2355-2361