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AN INFLUENCE OF pH ON STAPHYLOCOCCAL BIOFILM FORMATION

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The study of the influence of the non-specific external factor – the acidity of the medium, revealed the dependence of the film forming processes on the intensity of the factor and time of it action. ***The aim*** *of the research was to study an influence of acidity of nutrient media on biofilm formation of isolates of S. aureus and S. epidermidis.* ***Methods.*** *Isolates of S. aureus (n=7) and S. epidermidis (n=20) were inoculated on media with different pH (from 4.0 to 8.0) and cultivated 72 h. After 24, 48 and 72 h of cultivation the quantity of living cells was determined.* ***Results.*** *It has been shown that the maximal increase of cell number in films of S. aureus and S. epidermidis occurred in media with neutral pH, as well as the growth rates were closer to the maximum when the film was grown at pH 6 and 8. Thus, at neutral pH, the maximal cell growth took place for 72 h, when the number of cells in S. aureus film was 9.52±8.56 lg CFU / ml and S. epidermidis was 9.29±8.07 lg CFU / ml. At pH 4 and 5, the intensity of the growth of the culture was the smallest: the maximal cell count in the film did not exceed 5.5 lg CFU / ml.* ***Conclusion.*** *The strains of S. aureus were more resistant to the influence of acidity of medium, compare to S. epidermidis isolates.*

Keywords: biofilm formation, pH, staphylococci.

It's wide known that each species of microbe has its own characteristic range of pH values in which it grows and reproduces best. All bacteria are sensitive to the hydrogen ion concentration they find in their environment [1]. Most bacteria grow best around neutral pH values (6.5 – 7.0), but some can grow in very acid conditions and can even tolerate a pH as low as 2.0. Such microorganisms are called acidophiles. Even though they can live in very acid environments, their internal pH is much closer to neutral values. Some microorganisms can grow in alcaliphilic condition (pH value more than 9.0). They are called alcaliphiles [2].

Some bacteria produce acid during development of their culture, for example *Streptococcus* spp., *Lactobacillus* spp., *Actinomyces* spp., *Thiobacillus* spp., *Clostridium* spp. This acid is excreted and lowers the pH of the surrounding environment [1, 3]. In some cases, this eventually brings bacterial growth to a halt unless something else in the environment neutralizes the bacterial acid [4]. But there are microorganisms that resistant to lower pH values. Among mechanisms of this type of resistance ability to biofilm formation can be called. More over pH in biofilm may be lower than in surrounding environment [5, 6]. Usually the value of acidity of media is one of the limiting factors of bacteria growth. But in biofilm microorganisms become more resistant to environmental factors. Biofilms are surface-associated communities of

microorganisms enclosed within an extracellular polysaccharide, protein or composite matrix, which protect cells against environmental factors [7]. There are bacteria (for example streptococci) that have lower pH value in biofilm compare to planktonic culture [8, 9].

One of the most known bacteria able to form biofilm are staphylococci. These bacteria caused different types of pathological lesions of human [10], that's why possibilities of inhibition of their growth are actual questions of study. Staphylococci are typical neutrophiles and pH may play a significant role in development of their culture [11].

The aim of the research was to study an influence of different pH (from 4.0 to 8.0) on biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates.

Materials and methods. Research was done on the basis of Educational and Scientific Laboratory of the Department of Modern Technologies of Diagnostic and Treatment Process of Oles Honchar Dnipro National University (Dnipro, Ukraine). Strains of staphylococci obtained from vagina of women with microbiota disorders: *Staphylococcus aureus* (n=7) and *Staphylococcus epidermidis* (n = 20). Identification of bacteria based on characteristics, described in Bergey's manual [12] with use of ApiStaph test-systeme (bioMérieux, France).

Biofilm growth investigated in 96-well flat-bottomed plate in which the cell suspension added [13, 14]. From daily cultures of identified strains were prepared cell suspension, contained $1,5 \times 10^5$ colony forming units / mL (CFU / mL) with visual control by standard of turbidity MacFarland 0.5. In each well added 100 µl of culture medium (meat-peptonic broth, MPB) and 50 µl of the bacterial suspension. For the counting of the total cell amount, the aliquots (100 µ) of resuspended cells sowed on meat-peptonic agar (MPA) and incubated 24 h at 37 ºC. To monitor the dynamics of biofilm growth, samples were taken from individual wells in 24, 48 and 72 h. Biofilm removed from the wells using a bacterial loop and transferred to a test tube containing 2 ml of sterile saline. Samples were sterile homogenised and sowed on MPA. The number of colonies was the marker of biofilm growth effectiveness.

For the experiment using nutrient media with different pH (from 4.0 to 8.0). Media were prepared on basis of MPB with Na_2HPO_4-NaH_2PO_4 buffer (pH 6.0, 7.0, 8.0). Phosphate buffer (0.1 M) was prepared on the basis of one- and dibasic sodium phosphate in certain proportions (table 1). The medium with pH 4.0 and 5.0 were prepared without the addition of buffer solutions with commercial dry MPB according to instructions.

Statistical analysis of the results was made by standard statistical methods ($P<0.05$). Statistical analysis of the data was done by tools of MS Word.

Table 1

Components of phosphate buffer to prepare media

pH	0,2M Na_2HPO_4, ml	0,2M NaH_2PO_4, ml
6.0	12.3	87.7
7.0	61.0	39.0
8.0	94.7	5.3

Results. The results of the effect of acidity on the formation of biofilms by strains *S. aureus* during growing of biofilm along 72 h at different pH (as determined by the amount of CFU / ml) are shown in Fig. 1.

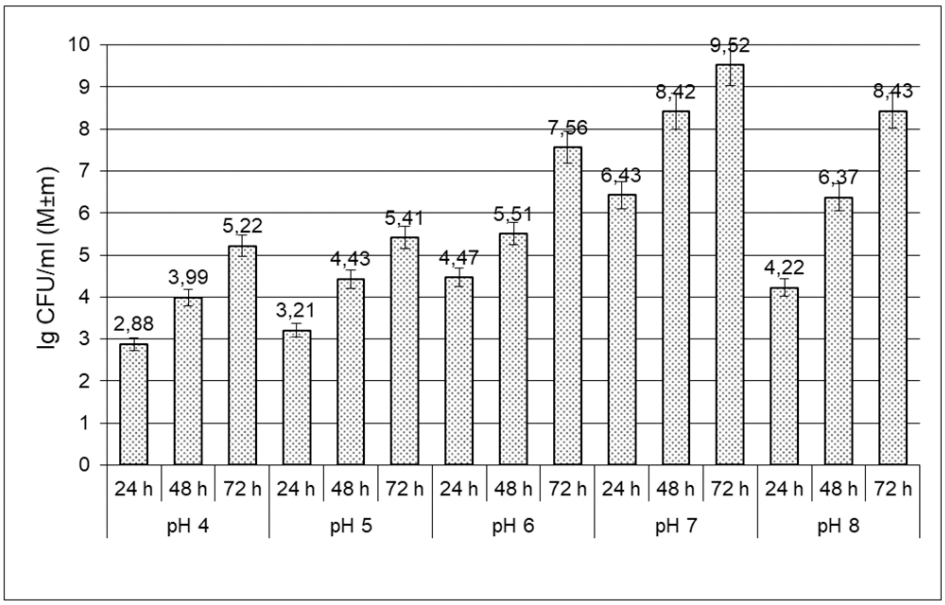

Fig. 1 Formation of *S. aureus* biofilms at different pH

From the presented data, it is seen that the maximum increase of the number of cells in the film occurred during cultivation at the neutral pH, although the formation of biofilm was observed for lower and high values of the acidity of the medium.

Thus, the maximum growth of cells in the film for all cases of cultivation took place for 72 h, however, the intensity of the increase of the number of cells in all cases was different. At pH 7.0, for 24 h, the number of cells was 6.43 ± ± 5.34 lg CFU / ml, which exceeded the values for other cultivation conditions by more than 90 times and in this time point amount of cells was even higher than the indices for 48 h of cultivation at a pH interval 4.0 – 6.0 and 8.0. These results indicated that pH 7.0 is the optimum conditions for cultivating of the *S. aureus* biofilm. The highest intensity of cell growth at pH 7.0 was observed from 24 to 48 h, when the number of cells increased by 97.7 times.

The growth rates of the film in medium with another pH were somewhat less intense, however, there was a clear film formation during the experimental period of time. Thus, at pH 8.0, a slightly more intense accumulation of cells was observed than at a pH of 6.0 for 48–72 h. At 24 h, the indices were close and at pH 6.0 and 8.0, respectively, were 4.47 ± 3.74 and 4.22 ± 3.48 lg CFU / ml. Then more intensive growth rates were observed for the culture grown in a medium with pH 8.0: for 48 h, the number of cells increased in 141 times compared with 24 h, and for 72 h – in 114 times compared to the previous time point. When cultivated in a medium with pH 6, studied markers were in 11 times and in 112 times higher respectively.

Compared with cultivation in medium with pH 7.0, the maximum number of cells after 72 h of cultivation at pH 6.0 was lower in 135 times and at pH 8.0 – in 15 times.

At pH 4.0 and 5.0 the intensity of the growth of culture was minimal: the formation of separate fragments of biofilm was fixed after 72 h. At pH 4.0, the number of cells after 24 h of cultivation was 2.88 ± 2.3 lg CFU / ml, and at pH 5.0 – 3.21 ± 2.51 lg CFU / ml. Further growth rates were close in intensity: after every 24 h the number of cells increased by about 10 times approximately. The maximum amount of cells at 72 h reached lg 5 levels: at pH 4.0 and 5.0 – 5.22 ± 4.66 and 5.41 ± 4.59 lg CFU / ml respectively, which exceeded 1.3×10^4 times was lower than the similar indicator for medium with pH 7.0.

The number of cells in *S. epidermidis* biofilm was studied during the 72 h cultivation in media with different pH (from 4.0 to 8.0) (Fig. 2). From the obtained results it can be seen that for *S. epidermidis*, as well as for *S. aureus*, the optimum pH of the culture medium is pH 7.0. In this case the most intense accumulation of cell mass in the film occurred.

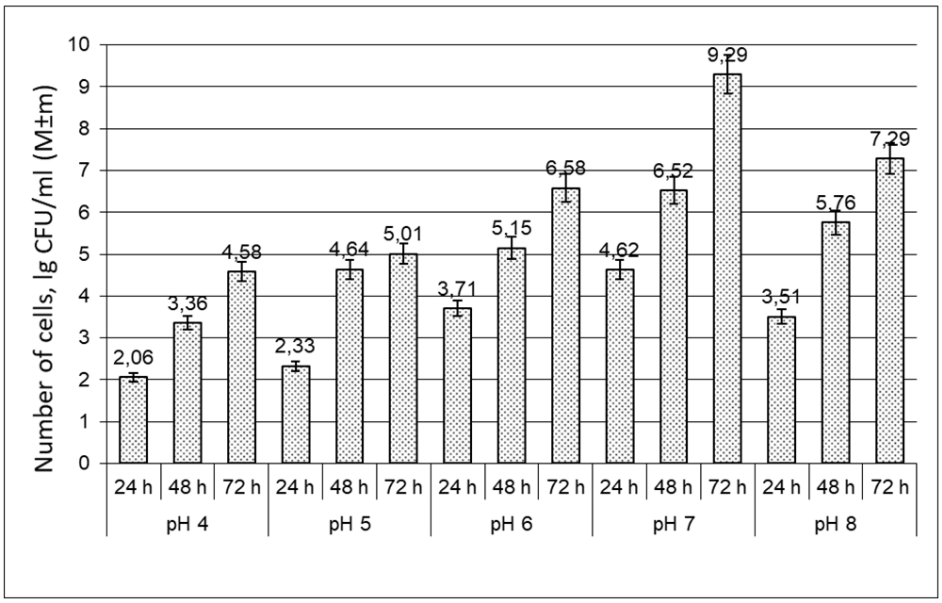

Fig. 2. Formation of *S. epidermidis* biofilm during cultivation at different pH for 72 h

Consequently, for 24 h of cultivation at neutral pH, the number of cells was 356.5 times higher than the number of cells in a biofilm that was formed in a medium with pH equal to 4.0. During cultivation at pH 5.0, the number of cells was lower by 195.2 times, at pH 6.0 – by 8.03 times, at pH 8.0 – by 12.8 times compared with the number of cells in a biofilm that formed in the medium at a neutral pH at the same time point.

During the first 24 h of cultivation, a significant increase in the number of cells was observed, which for all investigated ranges exceeded 100 times approximately. Subsequently (from 48 to 72 h), the growth rate of cells decreased in media with pH 4.0 and 5.0, not exceeding 10 times. The most prominent was cell growth at the neutral pH: 588 times for 72 h compared to 48 h.

At 48 h of cultivation, the number of cells in medium with a pH 4.0 was less than 1434.7 times, the amount of CFU at pH 5.0 was lower at 75.86 times, with pH 6.0 – 23.44 times, and at pH 8.0 – 5.78 times compared with the number of cells in biofilm, which was formed in a medium with a pH 7.0.

For 72 h of cultivation at pH 4.0 the number of cells in the film was lower at $5{,}12 \times 10^4$ times, at pH 5.0 – $1{,}90 \times 10^4$ times, at pH 6.0 – 512 times, at pH 8.0 – 100 times comparatively with the amount of CFU / ml in biofilms that were formed in a medium at a neutral pH.

Discussion. The results can be explained by the fact that staphylococci have one of the strongest cell walls among microorganisms [15], which withstands the influence of various external factors much longer than other bacteria. In addition, the effect of the acidity of the medium may be partially leveled due to the bigger matrix layer, which was more intensively produced by strains of *S. aureus*, while the films of *S. epidermidis* contained a larger number of cells.

It should be noted that pH is one of the factors that has an effect on the adhesion of bacterial cells to the surface [16], which is a species-specific feature and can have a decisive influence on the transition from planktonic form to biofilm. For example, the number of adhesive bacteria of the genus *Flexibacter* increases with decreasing pH of the medium. In the case of *Enterobacter cloacae* and *Chromobacterium*, the optimum pH value is within the range of 5.5–7.0. In the case where the pH is lower or higher, the number of adhesive cells is minimal. Maximum adhesion of *Pseudomonas fluorescens* occurs at pH 7.0. In turn, with the sharp increase in pH to extreme values, the formation of the *Archaeoglobus fulgidus* biofilm takes place. In our experiments, it was found that the optimum pH for the formation of the film is 7.0, with the inclusion of the optimum zone as the limit values of pH 6.0 and 8.0 in the optimum zone. That is, formation of a film with staphylococci is possible both at elevated and at lower pH, which in particular is typical for biotopes of a human organism, most of them have weakly acid or alkaline pH.

Investigating the influence of pH level of media on biofilm growth may also be of some curiosity given that antibiotics may be in ionized or non-ionized forms at different levels of acidity, and thus exhibit or not exhibit activity [17, 18], which can also be used to develop biofilm control measures.

Thus, the highest biomass of biofilms was recorded at a neutral pH that is close to the pH of the natural biotopes of staphylococcal strains. At the same time, the formation of biofilm occurred at different pH values, both acidic and alkaline. This can be considered as an important physiological peculiarity of staphylococcal metabolism, which contributes to the formation of biofilms with extreme values of the acidity of the medium.

Comparing the results of studies on the peculiarities of the formation of biofilms by the strains of *S. aureus* and *S. epidermidis* during their cultivation in media with different pH, it can be noted that strains of *S. aureus* were significantly more resistant to the influence of this factor, in particular at pH 6.0 and 8.0, relative to the neutral medium. In these conditions, an intensive accumulation of the cell mass of the film took place within 72 h, and it can be noted that the pH 8.0 had a lower effect on the growth of the culture. Under cultivation conditions at pH 4.0 and 5.0, the culture of both *S. aureus* and

S. epidermidis did not differ in the high efficiency of cell accumulation in the film, indicating close results of the number of cells.

ВПЛИВ pH НА ФОРМУВАННЯ БІОПЛІВКИ СТАФІЛОКОКАМИ

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Резюме

Вивчення впливу неспецифічного зовнішнього фактора – кислотності середовища – дозволило виявити залежність процесів формування біоплівки від інтенсивності та часу дії фактора. **Метою** дослідження було вивчення впливу кислотності поживних середовищ на формування біоплівки ізолятами *S. aureus* та *S. epidermidis*. **Методи.** Ізоляти *S. aureus* (n = 7) та *S. epidermidis* (n = 20) вирощували на середовищах з різним pH (від 4,0 до 8,0) і культивували 72 год. Через 24, 48 та 72 год культивування визначали кількість життєздатних клітин. **Результати.** Показано, що максимальне збільшення кількості клітин у плівках *S. aureus* і *S. epidermidis* відбувалося в середовищі з нейтральним pH, а темпи приросту кількості клітин були ближчі до максимуму при вирощуванні плівки при pH 6,0 та 8,0. За нейтрального pH максимальна кількість клітин визначалася через 72 год культивування і становила у плівці *S. aureus* 9,52 ± 8,56 lg КУО / мл, а у плівці *S. epidermidis* – 9,29 ± 8,07 lg КУО / мл. При pH 4,0 і 5,0 інтенсивність росту культури була найменшою: максимальна кількість клітин у плівці не перевищувала 5,5 lg КУО / мл. **Висновок.** Ізоляти *S. aureus* були більш стійкі до змін кислотності середовища порівняно з ізолятами *S. epidermidis*.

Ключові слова: формування біоплівки, pH, стафілококи.

ВЛИЯНИЕ pH НА ФОРМИРОВАНИЕ БИОПЛЕНКИ СТАФИЛОКОККАМИ

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Резюме

Изучение влияния неспецифического внешнего фактора – кислотности среды – позволило выявить зависимость процессов формирования биопленки от интенсивности и времени действия фактора. **Целью** исследования было изучение влияния кислотности питательных сред на формирование биопленки изолятами *S. aureus* и *S. epidermidis*. **Методы.** Изоляты *S. aureus* (n = 7) и *S. epidermidis* (n = 20) выращивали на средах с различными pH (от 4,0 до 8,0) и культивировали 72 ч. Через 24, 48 и 72 ч культивирования определяли количество жизнеспособных клеток. **Результаты.** Показано, что максимальное увеличение количества клеток в биопленках *S. aureus* и *S. epidermidis* проходило в среде с нейтральным pH, а темпы прироста количества

клеток были ближе к максимуму при выращивании пленки при pH 6,0 и 8,0. При нейтральном pH максимальное количество клеток определялось через 72 ч культивирования и составляло в пленке *S. aureus* 9,52 ± 8,56 lg КОЕ / мл, а в пленке *S. epidermidis* – 9,29 ± 8,07 lg КОЕ / мл. При pH 4,0 и 5,0 интенсивность роста культуры была наименьшей: максимальное количество клеток в пленке не превышало 5,5 lg КОЕ / мл. **Выводы.** Изоляты *S. aureus* были более устойчивы к изменению кислотности среды по сравнению с изолятами *S. epidermidis*.

Ключевые слова: формирование биопленки, pH, стафилококки.

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