



# Structure and composition of mono- and multispecies skin and environmental bacteria: effects of cosmetics and other biologically active compounds

Andrei Gannesen

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# THÈSE EN CO - TUTELLE INTERNATIONALE

Pour obtenir le diplôme de doctorat

Spécialité Aspect moléculaire et cellulaire de la biologie

Préparée au sein de l'Université de Rouen Normandie  
de l'Université d'Etat Lomonosov de Moscou et de l'Institut de microbiologie de l'académie des sciences de Russie.

**Structure et composition de biofilms mono- et multispécies de bactéries cutanées et environnementales: effets des cosmétiques et de certains autres composés biologiquement actifs**

Présentée et soutenue par

Thèse soutenue publiquement le 04 décembre 2018  
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## ***List of abbreviations***

AHL – acyl homoserine lactone  
ANP – A-type natriuretic peptide  
BNP – B-type natriuretic peptide  
CAMP-factor (CAMP-фактор) - Christie–Atkins–Munch-Petersen factor  
CFU – colony-forming unit  
CLSM – confocal laser scanning microscopy  
CNP – C-type natriuretic peptide  
GABA – gamma-amino butyric acid  
ID – inhibition dose  
LB – lysogenic broth  
MIC – minimal inhibitory concentration  
MQ-water – water cleaned with MilliQ system  
MRSA – methicillin-resistant *Staphylococcus aureus*  
MRSE – methicillin-resistant *Staphylococcus epidermidis*  
NUP – natriuretic peptide  
OD – optical density  
PNAG – 6-poly-N-acetyl-glucosamine  
PS – physiological saline  
PS291® - polysaccharide 291® or teflose  
QS – quorum sensing  
RCM – reinforced clostridial medium  
TSA – tryptic soy agar  
TSB – tryptic soy broth  
UTW™ – Uriage™ thermal water



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## ***General introduction***

Humans skin is the biggest organ where hundreds of microbial genera coexist together (Grice et al., 2009). Such coexisting was developed during evolution process: the host organism does not impede incoming and proliferation of commensal microbes, but fights against pathogens (including with help of commensal microbiota, Chiller et al., 2001). Microbial species ratio in human skin depends on sex, age, geographical region etc., but the general regularities inside a single group are similar (Grice et al., 2009; SanMiguel & Grice, 2015). One of important factors for microbial growth is microenvironment, which is based on physiological skin features. Normally three types of skin areas and microenvironment – sebaceous (oily), moist and dry are distinguished depending on the location of skin area, number of sebaceous and sweat gland on it (Grice et al., 2009). Each type of microenvironment is characterized by its own special set of microorganisms. In moist and sebaceous sites Actinobacteria (and especially former genus *Propionibacterium*) and Firmicutes are dominant, and in dry areas – Betaproteobacteria and Corynebacteria (Grice et al., 2009; SanMiguel & Grice, 2015).

Health of the human skin and skin microbiota status are tightly interrelated. Different skin

injuries and diseases (physical traumas, atopic dermatitis, psoriasis, other immune system disorders) lead to changes in microbial species ratio in skin (SanMiguel & Grice, 2015). This results in complications (overgrowth of pathogens, inflammations etc., Hong et al., 2011). Skin is a barrier which interacts permanently with environment and undergoes effects of exogenous and endogenous factors. These effects can impact also skin microbiota. Endogenous factors are determined by humoral and nervous regulation of skin state. Exogenous factors can be divided on biological (contacts with other living organisms), physical (air, moistening, environmental temperature, radiation etc.) and chemical (effects of different artificial or natural compounds). As chemical factors, in fact, we can classify all chemical compounds which interact with human skin, including drugs, antibiotics and cosmetics, which are used widely all around the world. Impact of these compounds on skin is very significant (Feuilloley & Orange, 2018).

Microbial biofilms are spatially and metabolically structured communities which are embedded in an extracellular polymeric matrix and usually located on different interfaces (Nikolayev & Plakunov, 2007). Biofilms are the most common life manner of microorganisms on Earth (Nozhevnikova et al., 2017). Biofilms can grow on different interfaces: liquid-air, solid object – air, liquid-liquid and solid object - liquid (Haussler, Fuqua. 2012). Biofilm formation is a universal property of bacteria, biofilms are found virtually everywhere on all artificial or natural surface (Vlamakis et al., 2013). In biofilms cells undergo physical and biological impacts of different kind in comparison with planktonic forms. In biofilm there are sharp gradients of concentration of nutrients, gases and ions, close adjacency of cells leads to their physical contacts, interchange of signal molecules and metabolites. All these phenomena determine special features of biofilms (Nikolayev & Plakunov, 2007).

When biofilms grow, the specific biofilm phenotype is developed (Nikolayev & Plakunov, 2007), when expression level of different genes is changed, which leads to expression of special cell features in comparison with planktonic state (for example, synthesis of the matrix). As a result, biofilms acquires properties close to multicellular organism. Some time ago there was the hypothesis about interkingdom multispecies biofilms of amoebas and bacteria as ancestors of multicellular organisms – sponges (Verhoeven et al., 2010). However, this hypothesis was incorrect: bacteria in biofilms have biofilm phenotype, but not genotype. Gene expression changes are not irreversible, cells can leave biofilms and return into planktonic state, so, there is no true cell differentiation like in multicellular organisms. But, actually, cells of one species inside a biofilm can differ significantly in their gene expression (Boles, Singh, 2008).

There are three general stages of biofilm formation: cell adhesion, growth and dispersal (Plakunov et al., 2017). In its turn, on each stage there are several sub-stages. On the stage of cell adhesion there are sub-stages of reversible (initial) and irreversible adhesion (Nikolayev &

Plakunov, 2007; Dutta et al., 2012; Ribeiro et al., 2012). Cells during initial adhesion step can navigate the surface with flagella and pili to find an optimal place. When irreversible adhesion starts, cells lose their motility (Romeo, 2008; Harmsen et al., 2010; Dutta et al., 2012). On growth stage synthesis of matrix and settlement of secondary colonizers occur (Nikolayev & Plakunov, 2007; Vlamakis, 2013). These processes can go simultaneously or step by step, so it is possible to combine them in one stage. After, biofilm enters the stage of maturation (Nikolayev & Plakunov, 2007; Mann, Wozniak, 2012; Vlamakis, 2013). On this stage complex three-dimensional structures can be formed like mushroom-like structures of *Pseudomonas aeruginosa* (Hansen et al., 2010) or round pellicles of *Bacillus subtilis* (Vlamakis, 2013). On dispersal step a part of cells loses their biofilm phenotype and returns into free planktonic state.

Quorum sensing should be mentioned specially. The term “quorum sensing” (QS) describes intercellular interactions in bacteria, which allow the community to coordinate genes expression in accordance with changes of population density (Camilli & Bassler, 2006). It is known that QS systems contribute biofilm formation, and lots of molecules serve as QS-factors in bacteria (Worthington et al, 2013). QS investigations in Gram-negative bacteria are conducted usually with use of *Vibrio fischeri*, *V. cholerae* and *P. aeruginosa* as objects (Worthington et al, 2013). Generally, QS-system work based on two proteins: the first synthesizes a signal molecule of autoinducer (AI) and the second protein is a receptor of autoinducer (Worthington et al, 2013). QS systems significantly contribute biofilm formation, and modern methods of biofilm eradication more often mean inhibition of QS, for example, with N-(2-pyrimidinyl)-butanamide (Kalia, 2015; Furiga et al., 2016; Leoni, Rampioni, 2018).

Microbial biofilms nowadays are an object of intensive research because of several reasons. The first is the interest of fundamental sciences to find out how normally monocellular bacteria can exist in multicellular communities. The second, biofilm formation is a serious problem in medicine and industry, because of their many-fold stronger resistance to antibiotics, immune system and biocides (Hansen et al., 2010) in comparison with planktonic cultures (planktonic cultures are totality of floating single microbial cells in liquid medium). Also, interesting (and dangerous) phenomenon is persisting cells – a small part of cell population in biofilm, which has lower metabolic activity and many-fold higher resistance to antibiotics than other biofilm cells (Plakunov et al., 2010). Persisting cells or persisters can reestablish the population after death of the most of cells after a strong negative impact (usually exogenous, like physical biofilm destruction or biocide attack), that makes persisters one of the factors of biofilm stability and resistance (Michiels, Fauvart, 2016).

Also, besides negative aspects, microbial biofilms conduct important processes like, for example, destruction of biodegradation-resistant pollutants of soils and wastewaters (Perni et al.,

2013). The fundamental problem for science is understanding how bacteria develop their so called group-level properties (Nadell et al., 2013). These properties are spatial structure of biofilms and coordination of cell behavior during interaction with each other and surfaces (Nadell et al., 2013). Also intracellular and intercellular metabolic and signal pathways in biofilms are of scientific interest.

To generalize, it should be stated that study of biofilms is one of priority areas of modern microbiology, and not only monospecies, but also multispecies biofilms. Human microbiota and its biofilms in human body are of special interest because of human microbiota and particularly skin microbiota in many respects determines the state and functioning of different systems of human organism (Thomas et al., 2017). So, study of biofilms of skin commensal, closely related to them microorganisms and how different active compounds affect them (what is the goal of present thesis) is actual. First, as mentioned above, it is interesting for fundamental science to investigate biofilms and their interactions with human organism. Second, it is actual for applied sciences: biotechnology, cosmetology and pharmacology. In cosmetology nowadays there is growing interest in new cosmetic compounds because of progressive rejection of preservative agents and deepening interrelation between cosmetology, dermatology and microbiology. The last requires developing of new compounds to maintain healthy skin microbiota. In pharmacology now search of new antibiofilm agents to combine with already existing antibiotics is actual. In biotechnology it is necessary to control biofilm growth in processes of biosynthesis of needful compounds or in maintaining of important biochemical process (like, for example, it is now performed in wastewater treatment process with anammox bacteria (Nozhevnikova et al., 2015)). Also, it is necessary to investigate not only effects of different active compounds on biofilms, but also biofilm structure, especially biofilm matrix, which is a strong barrier for different active compounds (Zhurina et al., 2014). Information about matrix structure is important for searching of compounds which are able to pass through it and reach cells inside biofilms.

## ***Key points of the thesis***

### **The aim of the work**

The aim of this work was to investigate effects of active compounds of different types on human skin commensal bacteria and their close relatives of natural environments, their monospecies and binary biofilms and investigate probable mechanisms of action of used compounds.

To reach the aim, several **tasks** were set and performed:

- 1) Investigate effects of antibiotic azithromycin on culture and biofilm growth of

*Pseudomonas chlororaphis* – nonpathogenic microorganism closely related with pathogenic *P. aeruginosa*. Azithromycin is an antibiotic widely used in treatment of *pseudomonas* infections.

2) Investigate effects of antihelminthic drug niclosamide on cultures and biofilms of human skin commensal bacteria *Micrococcus luteus*, *Kytococcus schroeteri* and *Staphylococcus aureus*. Find out if niclosamide can strengthen the effect of azithromycin.

3) Investigate effects of thermal water Uriage™ (TWU) and oligosaccharide PS291® on growth of monospecies and binary planktonic cultures and biofilms of human skin commensal bacteria *S. aureus*, *S. epidermidis* and acneic strains of *Cutibacterium acnes*.

4) Investigate effects of natriuretic peptides A- and C-type on growth of monospecies and binary planktonic cultures and biofilms of *S. aureus* and acneic strains of *C. acnes*.

5) Investigate biochemical composition of biofilm matrix of acneic strain *Cutibacterium acnes* HL043PA2 (RT5).

## Scientific novelty of investigations

It was unveiled that subinhibitory concentrations of azithromycin stimulate growth of *P. chlororaphis*, and acylhomoserine lactone-dependent QS-system is involved in this process. It was unveiled that azithromycin affects *P. chlororaphis* in a special way: biofilm growth stimulation appears at relatively higher concentrations, while at lower concentrations (several orders lower than minimal inhibitory concentration (MIC)) there is inhibition of biofilm growth. It was shown for the first time also that azithromycin-stimulated biofilms of *P. chlororaphis* have bigger amount of polysaccharide component of matrix in biofilms and more resistant to heat shock.

Strong inhibitory effects of niclosamide on planktonic cultures and biofilms of *S. aureus*, *M. luteus* and *K. schroeteri*. It was shown that niclosamide quenches stimulatory effect of subinhibitory concentrations of azithromycin on biofilms of *S. aureus*. This makes niclosamide a promising antibiofilm agent.

It was shown for the first time that TWU™ and PS291® significantly decrease biofilm growth of *S. aureus* and *C. acnes* without toxic impact on cells. Probably, these cosmetic compounds modify cell adhesion ability, and this makes them a perspective base for new cosmetics development.

It was unveiled that human natriuretic peptides (NUP) have strong effect on biofilms of Gram-positive human skin commensal bacteris. It was established that effects of NUPs A- and C-type on biofilms of *S. aureus*, *S. epidermidis* and *C. acnes* depends significantly on growth conditions that can correlate with different skin status and NUP regulatory functions: enlarge competitive features of *C. acnes* and *S. epidermidis* against *S. aureus*. This approves the suggestion

that NUPs have capacity to affect the microorganisms and co-evolution of human skin microbiome and human regulatory molecules. Also it makes NUPs perspective for application in dermatology and cosmetology as normalizing agents for microbiota.

Deep biochemical investigation of biofilm matrix of *C. acnes* was done for the first time. It was shown that, for investigated strain, the polysaccharides were dominant matrix component. Total proteome analysis of matrix was performed for the first time, more than 400 proteins were shown to be a part of matrix, their functions were suggested. SERS-spectroscopy of matrix and biofilms biomass of *C. acnes* was made for the first time, spectral fingerprints were obtained.

## **Practical implications of the work**

The universality of the phenomenon of biofilm growth stimulation by antibiotics was shown: azithromycin stimulates growth of biofilms of not only human pathogenic bacteria, but also of soil saprotrophic bacteria. The involvement of QS-system of *P. chlororaphis* in growth stimulation was shown, this approves good perspectives of QS-quenching agents (especially AHL-dependent QS) in biofilm eradication treatments and quenching of antibiotic-mediated biofilm stimulation. It was shown that in stimulated biofilms, polysaccharidic components of matrix are synthesized more actively, and probably because of that azithromycin-stimulated biofilms are more resistant to heat shock. This is useful for further research.

Good perspectives of niclosamide as potential antibiofilm agent for Gram-positive were shown. At human non-toxic concentrations, it eradicates efficiently biofilms of Gram-positive commensal bacteria, and also quenches stimulatory effect of azithromycin on *S. aureus* biofilm. Niclosamide can be used in the future in complex antibiofilm dtugs.

TWU<sup>TM</sup> and PS291<sup>®</sup> are shown to be perspective base components for novel cosmetics to fight agains biofilms of *S. aureus* and *C. acnes* in human skin.

NUPs were shown perspective for use in antibacterial treatment, also the new property of NUPs was shown: ability to regulate skin microbiota. Before this ability was considered as uncharacteristic for NUPs, and this gives broad opportunities for endocrinology and clinical practice.

The robust method of Gram-positive bacteria biofilm matrix extraction was developed. This method is non-chemical, this allows to preserve compounds of matrix unchanged for deep analysis.

Protein composition of matrix was revealed. It will be used in further investigation of *C. acnes* pathogenicity.

## States for defense

1) Azithromycin at extra-low concentrations inhibits growth of *P. chlororaphis* 449 biofilms. At higher concentrations stimulation occurs, which disappears with approach to MIC. Stimulation and inhibition also result in increasing and decreasing of the polysaccharide moiety of the matrix. Biofilms of *P. chlororaphis* which were grown in the presence of stimulating concentration of azithromycin are more tolerant to heat shock than in control without azithromycin. AHL-dependent QS-system is involved in stimulation process: expression of a plasmidic gene encoding for AHL-lactonase totally abolishes the stimulatory effect of azithromycin in genetically modified strains of *P. chlororaphis*.

2) Niclosamide already at concentration 0.1 µg/ml strongly inhibits the growth of metabolically active cells of *S. aureus*, *M. luteus* and *K. schroeteri*. At 1 µg/ml, niclosamide in a medium fully inhibits growth of metabolically active bacteria in biofilms. In combination with azithromycin niclosamide neutralizes the stimulating effect of azithromycin on *S. aureus* biofilms.

3) UTW™ and PS291®, probably because of modification of cell adhesion ability, inhibit efficiently biofilm growth of *C. acnes* and *S. aureus*. The effect of thermal water should be correlated to its chemical composition. Moreover, UTW™ and PS291® are able to regulate skin microbiota balance: due to decreasing of competitive advantages of *S. aureus*, both cosmetics increase the ratio of *C. acnes* and *S. epidermidis* in binary biofilms with *S. aureus*.

4) NUPs A- and C-type affect *C. acnes*, *S. epidermidis* and *S. aureus*, and NUP effects depends on cultivation conditions. This suggests different roles of NUPs in different conditions in skin. NUPs possess regulatory functions and they are able to modulate the CFU balance of microorganisms in binary biofilms.

5) A mean of 54% of the matrix organic matter of *C. acnes* RT5 biofilms is formed by polysaccharides. Proteins and DNA account for 16% of the organic matrix. About 30% of the matrix organic compounds are molecules characterized as non-peptidic, non-carbohydrate and non-DNA. These molecules are low-molecular metabolites including porphyrins and intermediates of porphyrin synthesis.

6) 447 different proteins were found in the matrix of *C. acnes* RT5. More than 20 hydrolases of different substrate specificity, more than 40 proteins of unknown origin and function, a lot of enzymes and structural proteins. Most of the proteins probably appear in the matrix after cell autolysis during biofilm formation. Hydrolases should be involved in the pathogenicity of *C. acnes* biofilms in skin and other microenvironments in the human organism.

7) SERS investigation of matrix allowed to find 57 peaks of different intensity, which compose spectral fingerprint of *C. acnes* matrix. The same was done for *C. acnes* cells. All spectra



need to be deeply investigated by other methods, but presently they will be used in united database of bacterial SERS spectra construction.

## **Thesis approbation**

Results of the thesis were presented in 11 scientific congresses and conferences:

- 1) III All-Russian scientific and practical conference "The process of life development within abiotic changes on the Earth" (23-30 September 2014, Listvyanka);
- 2) XXVII Winter school for young scientists "Perspective trends of physico-chemical biology and biotechnology" (9-12<sup>th</sup> February 2015, Moscow);
- 3) VIII Moscow international congress "Biotechnology: state of the art and perspectives" (17-20<sup>th</sup> March, 2015, Moscow);
- 4) XXII International scientific conference of students, PhD-students and young scientists "Lomonosov-2015" (13-17<sup>th</sup> April, 2015);
- 5) X International school-conference for young scientists "Actual aspects of modern microbiology" (27-30<sup>th</sup> October, 2015, Moscow)
- 6) 4<sup>th</sup> International congress "Cosm'innov" (24-25<sup>th</sup> May, 2016, Orleans, France)
- 7) International congress "Antimicrobial resistance in microbial biofilms and options for the treatment" (5-7<sup>th</sup> October 2016, Gent, Belgium)
- 8) XI International school-conference for young scientists "Actual aspects of modern microbiology" (9-12<sup>th</sup> November, 2015, Moscow);
- 9) XXIX Winter school for young scientists "Perspective trends of physico-chemical biology and biotechnology" (7-10<sup>th</sup> February 2015, Moscow);
- 10) XXI International Puchshino school-conference for young scientists "Biology – the science of XXI century" (17-21<sup>th</sup> April, Puchshino);
- 11) 9th International Conference on Skin Ageing and Challenges (Porto, Portugal).

## **Publications**

6 scientific articles based on materials of the thesis were already published: 2 reviews and 4 experimental articles. All 6 are in journals indexed in Scopus and Web of Science, 3 are in journal of RSCI core. 2 experimental articles are in preparation for publication.

**Individual contribution of the thesis author.** The author conducted experimental studies, all results were obtained by author or with his direct participation. Names of co-authors

were noted in all publications and cases.

**Thesis structure.** The thesis consists in abbreviations list, bibliographic review, experimental part, conclusion and bibliographic references list.

## ***Bibliography review***

### **CHAPTER 1. Human skin microbial commensal microorganisms and microorganisms closely related with them, their biofilms and interactions with human organism**

#### **1.1. Human microbiota**

Human microbiota is a community of microorganisms (commensal and pathogens), which live inside and on the surface of human organism and ecologically interrelated with it and with each other (Thomas et al., 2017). The human evolved together with his microbiota (Thomas et al., 2017). Different authors evaluated differently the number of microbial cells in human organism and on its surface (data varies between  $10^{12}$  and  $10^{20}$  cells, Bianconi et al., 2013). Similarly, dispersion of data concerning the number of human organism own cells was great – several orders (Sender et al., 2016). Some scientists nowadays are in doubt with these data which are widespread in scientific literature. According to Rosner, 2014, the frequently cited ratio of microbial to human cells equal 10:1 origins from the article of Luckey, 1972, where the microbial cell number was estimated by their amount in gut contents. Some authors suppose that the data of this article and following are a partly incorrect because they were based on a too large generalization and averaging of the human body volumes, mass, microbial cell volumes and so on. (Sender et al., 2016). Results of modern metadata analyses, which include calculation of multiple parameters, suggest that in average the human body consists in  $3.72 \times 10^{13}$  (Bianconi et al., 2013). The number of microbial cells inside and on the surface of the human organism was estimated about  $3.9 \times 10^{13}$  (Sender et al., 2016), so the actual ratio may be about 1:1.

Nevertheless, the species biodiversity of the human microbiota is great: according to modern estimations, at least 40000 strains are associated with the human organism, these strains belong to 1800 genera and possess more than 9 million genes differing from human genes (Schwartz & Rusch, 2016). It is a 100-fold bigger amount of genes than in humans (Thomas et al., 2017). Also, the microbiota of each individual possesses an amount of genes which is 10-fold bigger than the proper individual's genome (Thomas et al., 2017). Microbiota of humans includes representatives of all three life domains – archaea, prokaryotes, eukaryotes (Thursby & Juge, 2017), and also viruses, which form the human virome (Virgin, 2014). The most complete data about the genetic diversity of the human microbiota to date were obtained by metagenomics and metatranscriptomics approaches, which have advantages (total DNA analysis allows to find non-cultivable microorganisms) and disadvantages (dependence on nucleic acids preparation methods, impossibility of complete microorganism description, Walker, 2016). However, microorganisms

cultivation methods of on different laboratory media nowadays allow to investigate a very limited amount of microbes, between 0.1 and 1% of the real population (Walker, 2016).

The human microbiota performs a number of important functions: synthesis of needful compounds (Lai et al., 2010; SanMiguel & Grice, 2015; Nakatsuji et al., 2018), defense against pathogens and immune system functioning support (SanMiguel & Grice, 2015). Close interaction of human microbiota with human organism during evolution processes formed finely-tuned and very complexly organized communities, that some authors call “the second brain” (Ochoa-Repáraz & Kasper, 2016), and human organism together with its microbiota – “superorganism” (Thomas et al., 2017).

## **1.2. Human skin microbiota**

Humans skin is the biggest organ where hundreds of microbial genera coexist together (Grice et al., 2009). Such coexistence was developed during the evolutionary process: the host organism does not impede incoming and proliferation of commensal microbes, but fights against pathogens (including with the help of the commensal microbiota, Chiller et al., 2001). The microbial species ratio in human skin depends on sex, age, geographical region and environment, but the regularities within a single group are generally similar (Grice et al., 2009; SanMiguel & Grice, 2015). One of the important factors for microbial growth is its microenvironment, which is depending on physiological skin features. Normally three types of skin areas and microenvironment are described – sebaceous (oily), moist and dry – following the location of the skin areas and the density of sebaceous or sweat glands (Grice et al., 2009). Each type of microenvironment is characterized by its own special set of microorganisms. In moist and sebaceous sites Actinobacteria (and especially former members of the genus *Propionibacterium*) and Firmicutes are dominant, whereas Betaproteobacteria and Corynebacteria are essentially found in dry areas (Grice et al., 2009; SanMiguel & Grice, 2015).

As the first barrier and the biggest human organ, skin is responsible for several important functions: thermoregulation, excretory function, defense function, gases exchange. Microorganisms which inhabit the human skin are involved in processes on skin and significantly determine its status (Achermann et al., 2014; Leung et al., 2018). Skin status changes (genetic distortions, diseases, exogenous factors) are generally leading to skin microbiota changes. For example, in atopic dermatitis a raucous proliferation of *S. aureus* on affected areas can occur, which results in disease complications (Elfatoiki et al., 2016; Lacey et al., 2016). Similarly *S. aureus* can overgrow in psoriasis (Totte et al., 2016) and folliculitis. When *S. aureus* starts to proliferate actively in hair follicles hollows it can rapidly cause inflammation (Balasubramanian et al., 2017). Dandruff is another skin disorder which, according to different estimations, it affects about half of the world population (Xu et al., 2016). Etiology of dandruff remains to date a matter of discussion, but

frequently fungi of the *Malassezia* genus are associated with it and proliferate intensively on affected skin areas. *Malassezia* fungi themselves are causative agents of tinea versicolor and sometimes proliferates abundantly on skin affected with atopic dermatitis and psoriasis (Xu et al., 2016). Also, *Malassezia* sp. is a cause of seborrhea (Soarez et al., 2015).

### **1.3. Some representatives of the human skin microbiota**

In this tip some microorganisms of human skin and closely related ones are described.

#### **1.3.1. *Pseudomonas***

*Pseudomonas* are not a bacterial genus classically associated to skin although metagenomic studies have shown that, even on healthy skin, in some moistly area they can account for more than 90% of the bacterial population (Grice et al., 2008). On the human skin, a lot of *Pseudomonas* species can be found, namely *P. fluorescens*, *P. putida* and *P. aeruginosa* are the most common. *P. aeruginosa* is a soil saprotroph (Deredjian et al., 2014), but it is much more well-known as a human pathogen (Loveday et al., 2014). Lots of studies on *P. aeruginosa* are already published, and lots of works are focused on it each year. That because *P. aeruginosa* is an opportunistic, fast growing and frequently aggressive pathogen. Its virulence is much higher than that of *P. fluorescens* and *P. putida*. It is involved and can be also the causative agent of a lot of diseases including lung cystic fibrosis (Fernandez et al., 2013), urinary tract infections (Tajbakhsh et al., 2015), artificial implants fouling (Cole et al., 2014) or otitis (Wang et al., 2005). Recently (before meticillin-resistant *S. aureus* abundant spread) *P. aeruginosa* was the main causative agent of bacteremias with highest mortality rate (Kreger et al., 1980). In terms of skin disorders, *P. aeruginosa* is responsible for the green nails syndrome (Chiriac et al., 2015), wounds infections and inflammations (Pfalzgraff et al., 2018), *pseudomonas*-caused folliculitis or hot tubes folliculitis (because of heating tubes in water pools and other water facilities are frequent sources of transmission, Fowler & Stege, 1990), ecthyma gangrenosum (Mull et al., 2000).... *P. aeruginosa* is able to destroy the skin structure in bacteremia which leads to serious exacerbations (Wu et al., 2011). *P. aeruginosa* is especially dangerous for people with compromised immunity (Bassetti et al., 2018). The difficulties of *P. aeruginosa* infections treatment are connected with its ability to form biofilms on skin, in particular, in wounds (Davis et al., 2008; Karna et al., 2016). Also, because of rather long-term fight against *P. aeruginosa* in clinics, dangerous multidrug resistant strains of *P. aeruginosa* appeared (Potron et al., 2015). Now *P. aeruginosa* is considered as the main infectious causative agent amongst Gram-negative rods (Bassetti et al., 2018).

Because of all these facts *P. aeruginosa* now is a model object for biofilm studies and studies of different antibacterial compound action on biofilms. *P. aeruginosa* is a very popular object, a number of monographs and lots of scientific articles every year are dedicated to it, so here only a very short review of the key points directly related to the present thesis will be made. Lots of works

are dedicated to *P. aeruginosa* biofilms resistance to antibiotics. For example, resistance to tobramycin and ciprofloxacin (Stewart et al., 2015), ceftazidime and colistin (Furiga et al., 2016) and other. A number of works are dedicated to azithromycin action on *P. aeruginosa* biofilms and its efficiency (for instance, Phelan et al., 2015, Saini et al., 2015). Concerning azithromycin resistance, MexAB-OrpM and MexCD-OprJ efflux pumps have been described for their role in resistance establishment (Gillis et al., 2005). Azithromycin also interacts with AHL-dependent QS-systems Las and Rhl in *P. aeruginosa* and blocks their functioning (Tateda et al., 2001), and by this way interferes with biofilm formation (Nalca et al., 2006). On *P. aeruginosa* a biofilm stimulatory effect of sub-MIC antibiotic concentrations was described. For example, doxycycline and polymyxin B in sub-MIC concentrations stimulate *P. aeruginosa* biofilms growth (Tote et al., 2009). Gentamicin in sub-MIC concentrations increases the expression of at least 30 genes (including the Lon protease) which are responsible for biofilm formation in *P. aeruginosa* (Marr et al., 2007).

A number of works concern the interactions of *P. aeruginosa* with signal molecules of the human organism. It was shown that catecholamines and  $\gamma$ -amino butyric acid (GABA) stimulate the growth of *P. aeruginosa* and increase its virulence (Lesouhaitier et al., 2009). Interferon- $\gamma$  and dynorphin can exert similar effects (Lesouhaitier et al., 2009). Recently it was shown that the genome of *P. aeruginosa* encodes for an enzyme, the amidase AmiC, which is an ortholog of the C-type natriuretic peptide (CNP) receptor (Rosay et al., 2015). This is probably a reason of CNP's ability to inhibit biofilm growth by *P. aeruginosa*. In *P. aeruginosa*, AmiC is a regulator of the amidase operon *ami* expression and therefore has a large pleiotropic activity (Wilson & Drew, 1991).

*P. putida* is a saprotrophic microorganism living in soil, water (Fernandez et al., 2015) and rhizosphere, that makes it useful in the context of plants protection against phytopathogens (Bernal et al., 2017). However, herewith *P. putida* can also behave as an opportunistic pathogen and can be a causative agent of nosocomial infections (Fernandez et al., 2015). *P. putida* can form biofilms in skin and change the structure of the epidermis (Fernandez et al., 2015). Despite of the fact that *P. putida* infections are rather rare and usually occur in patients with compromised immunity, there are many data about multidrug resistant strains of *P. putida* that can represent serious danger (Molino et al., 2014; Hardjo Lugito et al., 2015). For example, strains of *P. putida* have been now identified which are resistant to carbapenem, ciprofloxacin (Kumita et al., 2009), cefepime (Luczkiewicz et al., 2015) and some other beta-lactams (Trevino et al., 2010). There are described cases of abundant growth of *P. putida* in patients with diabetic gangrene and following exacerbations (Hardjo Lugito et al., 2015).

*P. fluorescens* is also a soil and water saprotroph, frequently found in the rhizosphere, which

can protect plants against phytopathogens (Kremmydas et al., 2013) and was even patented for crops treatment. However, as *P. putida*, *P. fluorescens* can colonize the human skin and be a cause of infections which include biofilm infections (Scales et al., 2014). Treatment of such infections can be difficult for multiple reasons and the principal is the high adaptability of this microorganism. For instance, different works describe the resistance of *P. fluorescens* resistance to biocides used as antiseptics: glutaraldehyde (Simoes et al., 2006), benzyldimethyldodecylammonium (Ferreira et al., 2011). Data concerning *P. fluorescens* antibiotic resistance are also abundant, in particular against beta-lactams (Bompard et al., 1988) and cephalosporins (Luczkiewicz et al., 2015). It was also shown that atmospheric pollutants, such as  $\text{NO}_2$ , can increase *P. fluorescens* resistance to ciprofloxacin and chloramphenicol through activation of MexEF-OprN efflux pumps genes expression (Kondakova et al., 2016). Moreover, it was shown on *P. fluorescens* that a range of endogenous molecules produced by the human organism can modulate the virulence and biofilm formation of this bacterium. These compounds can be antibacterial or traditionally considered as non-antibacterial. For example, substance P (a neuropeptide) increases the cytotoxicity of *P. fluorescens*, and in common with the hormone epinephrine (adrenaline) increases the swarming motility of the cells (Biaggini et al., 2015). The hormone serotonin also increases the swimming motility of *P. fluorescens* (Biaggini et al., 2015). Conversely, in some systems, the inhibitory neurotransmitter GABA inhibits the growth of *P. fluorescens* in biofilms (Dagorn et al., 2013). If concern antibacterial endogenous agents, it was shown recently that  $\beta$ -defensin-2 is able to increase the virulence of *P. fluorescens* cells (Madi et al., 2013). Thus, despite of the fact that *P. fluorescens* and *P. putida* are traditionally considered as safe rhizosphere microorganisms in comparison with *P. aeruginosa*, it seems to be that a number of molecules of human organism can interact with them. This can suggest at least universal mechanisms of interaction between microorganisms colonizing skin and human organism. Because of that it is interesting to investigate the effects of such active compounds and, in particular, antibiotics, on the growth of microorganisms which are closely related to widespread and well-known pathogens. For example, in a microorganism such as *Pseudomonas chlororaphis*.

*P. chlororaphis* is a rhizospheric saprotroph which is closely related to opportunistic *Pseudomonas* inhabiting the human skin – *P. aeruginosa* and *P. fluorescens* (Mishra et al., 2009; Hesse et al., 2018). In databases there are works dedicated to genetics and regulatory processes in *P. chlororaphis* biofilms. For instance, it was shown that functioning of phenazine synthesis system (Wang et al., 2016; Yu et al., 2018), regulatory system GacS/GacA (Li et al., 2015) and AHL-dependent QS-system (Peng et al., 2018) for biofilm formation in *P. chlororaphis*. However, at the time of present thesis preparation, there were no works found in the literature concerning the effects of active compounds (drugs etc.) on *P. chlororaphis*. Only Shepelevich et al. made a screening of *P.*

*chlororaphis* strains resistance to a range of antibiotics: non-effective ones were considered as perspective as herbicides which will not inhibit *P. chlororaphis* growth (Shepelevich et al., 2012). But authors did not examined the effects of antibiotics on *P. chlororaphis* biofilms, this makes present work original and actual.

### **1.3.2. *Micrococcus luteus***

*M. luteus* is a Gram-positive non-motile aerobic cocci, which form tetrads and belong to the phylum Actinobacteria (Hanafy et al., 2016). *M. luteus* is a saprotroph and a member of the normal human skin microbiota (Kloos et al., 1974, Daeschlein et al., 2012). It localizes mostly in the stratum corneum (Lange-Asschenfeldt et al., 2011), the upper surface of the skin formed of dehydrated cells containing an abundance of keratine and phospholipids. This microorganism is also found in water and soil (Mauclaire & Egli, 2010). The interest to *M. luteus* was arising at the end of XX century, when it was isolated from the human skin. Cases of *M. luteus*-caused bacteremia were described, but those cases were detected in people with health issues: in carcinoma (Albertson et al., 1978) and renal diseases (Peces et al., 1997). In the first case bacteremia occurred after surgical operation, and in the second one because of catheter infection; in both cases *M. luteus* was introduced into the weakened organism after exogenous surgical impact. After, a case of *M. luteus* mediated endocarditis was described in an old woman with compromised immunity. She underwent an operation for removing breast cancer, chemotherapy and course of drugs (Miltiados & Elisaf, 2011). Miltiados & Elisaf established in their work that, at the time they were writing the paper, only 17 cases of *M. luteus*-mediated endocarditis were described in the literature. They suggested that *M. luteus* was safe for healthy people, but nevertheless some investigators consider it as a potential opportunistic pathogen (Mauclaire & Egli, 2010). In 2010 the full genome of *M. luteus* was sequenced (Young et al., 2010), and it was shown that *M. luteus* belongs a the group of free-living microorganisms with the smallest genome (about 2.5 million b.p.). Small length of genome results in a range of metabolic limitations for *M. luteus*: for example, it is unable to growth on a medium with glucose as only carbon source. Authors suggest that such genome reduction could be an adaptation to the ecological niche of *M. luteus* in skin (Mauclaire & Egli, 2010). Nevertheless, it is generally considered that a small genome is an indication of potential pathogenic activity. Indeed, as they are unable to synthesize many necessary compounds for their metabolism, these bacteria need to scavenge host molecules with a risk of alteration of eukaryotic cells.

Biofilms of *M. luteus* are also poorly investigated. It seems that the little interest of researchers can be explained by the general neutrality of *M. luteus* if it is safe for healthy people. *M. luteus* was used as a model in Matsura et al. work where they studied biofilm growth in microfluidic system and the influence of medium pH-level (Matsura et al., 2013). Coumarins were shown to be active against *M. luteus* cultures (Emmadi et al., 2014), but the authors did not tested the activity of



coumarins against *M. luteus* biofilms. An interesting work was performed by Mauclaire & Egli (2010) where they compared biofilm formation and culture growth of *M. luteus* in microgravity in the ISS and compared to normal gravity conditions. They found that in microgravity *M. luteus* grows better in planktonic form and worse in biofilms in comparison with normal gravity. Also, on ISS the biofilm matrix of *M. luteus* contains less proteins and colloid polysaccharides, and the cell surface becomes more hydrophobic.

As other skin microbes, *M. luteus* can be affected by antibiotics, but in this area there are also little amount of works. There are some data about the activity of some quinolone antibiotics against *M. luteus*, which block the activity of the DNA-gyrase (Zweerink & Edison, 1986). There are also data about *M. luteus* isolated from human skin and containing the plasmid pMEC2, which encodes for resistance to macrolide antibiotics (Liebl et al., 2002). This plasmid encodes for a resistance factor of peptidic structure which has 50-54% of similarity with the ones of Gram-positive opportunistic pathogens with high G-C pairs amount – *Corynebacterium* and *Cutibacterium* (former *Propionibacterium*). Because of this, authors suggested a probable role of non-pathogenic *M. luteus* in horizontal transfer of antibiotic resistance genes. At the time of the present thesis preparation there no data were found concerning the potential interaction of *M. luteus* and endogenous human molecules.

*M. luteus* can be interesting for researchers as a potential biofuel source: it is potentially able to synthesize alkenes because of possession of a gene set for metabolism of amino and fatty acids (Young et al., 2010; Surger et al., 2018). However, the little interest to *M. luteus* in the context of medical research may be unwarranted. Indeed, it was shown that *M. luteus* isolated from poultry wastewaters possesses a keratinolytic activity (Laba et al., 2015). This is logical with the fact that this bacterium is living in the stratum corneum but it can also suggest a potential effect of *M. luteus* in skin by metabolizing keratin and it appears that the keratinolytic activity of micrococci can be potentially active in the stratum corneum. Also, cells of *M. luteus* can function as liposome-like capsules for chlorhexidine, which protects chlorhexidine from lysis inside neutrophils (Wendel et al., 2015). In this context, *M. luteus* was used as container for transporting chlorhexidine into neutrophils and temporary its conservation inside them. After *M. luteus* cell lysis chlorhexidine serves as an additional tool for neutrophil fighting against pathogens, for example, like *Fusobacterium cecrophorum*.

### **1.3.3. *Kytococcus schroeteri***

The genus *Kytococcus* was separated from the genus *Micrococcus* in 1995. *Kytococcus* differs from *M. luteus* by its phylogenetics, menaquinone composition (MQ 7,8,9 and 10 in *M. luteus* and MQ 8 and MQ 8-H<sub>2</sub> in *Kytococcus*), peptide bridges composition in peptidoglycan, polar lipids set and G-C ratio in the genome (*Kytococcus* – 68-69%, *M. luteus* – 70-76%, Stakebrandt et

al., 1995; Becker et al., 2002). This genus consists of three species - *K. schroeteri*, *K. sedentarius*, *K. aerolatus*, and the genus name can be translated as “skin cocci” (Chan et al., 2012). *K. schroeteri* was isolated in 2002 from blood of patient with endocarditis and named in honor of the German microbiologist and mycologist Joseph Schroeter (Becker et al., 2002). *K. schroeteri* lives on the human skin, but there were lots of described clinical cases of endocarditis after artificial heart valve implantation or heart shunt (Aepinus et al., 2008; Yousri et al., 2010; Schaumburg et al., 2013). Resistance to beta-lactams was noticed in *K. schroeteri* (Aepinus et al., 2008; Schaumburg et al., 2013). Cases of shoulder prosthetic devices infections and following exacerbations caused by *K. schroeteri* were described (Chan et al., 2012). Also, there were described cases of different bacteremia in patients with leukemia (pneumonia (Blennow et al., 2012; Amaraneni et al., 2015), skin ulcers and crusted papules (Nagler et al., 2011)). Papules and ulcers on skin caused by *K. schroeteri* seem to be logically explained by the close relation of *K. schroeteri* with *M. luteus*. *M. luteus* possess a keratinolytic activity (see above), and *K. schroeteri* probably also. As with *M. luteus*, problems caused by *K. schroeteri* normally appear in people with compromised immunity: after implantation or anti-cancer chemotherapy.

There were no found data concerning signal interaction of *K. schroeteri* and human organism, in particular, human skin at the time of present work preparing. Also there were no studies found concerning *K. schroeteri* biofilms and the effects of different active compounds on *K. schroeteri* biofilms. This makes present work actual and original.

#### **1.3.4. *Staphylococcus aureus***

*S. aureus* is a Gram-positive non-motile catalase-positive facultative anaerobic cocci (Masalha et al., 2001), which is able to perform nitrate and nitrite respiration (Balasubramanian et al., 2017). If *P. aeruginosa* is the most frequent Gram-negative causative agent of nosocomial and other infections, *S. aureus* is the most frequent Gram-positive infectious agent of humans, if not particularly the most widespread and frequent infection causative in general (Green et al., 2012). Because of this, of *S. aureus* fast growth and easy simplicity in work with and, as final, its lower danger in comparison to other pathogens like *Bacillus anthracis*, *S. aureus* is one of the model microorganisms for Gram-positive bacteria and their biofilms the more frequently employed. *S. aureus* causes 10-30 cases of bacteremia per 100000 citizens in developed countries per year (Tong et al., 2015). Infections caused by *S. aureus* can affect different organs and areas in the human organism including the respiratory tract and gut as well as artificial implants and catheters (Tong et al., 2015; Thippeswamy et al., 2011). For example, the colonization and biofilm formation of *S. aureus* on the surface of venous and hemodialysis catheters leads to sepsis, this in combination with the high resistance of *S. aureus* biofilms to antibacterials leads to complication of treatment process (Tran et al., 2012).

Despite of obvious danger for human health, a large percentage of the healthy population (up to 30% in adults and 70% in children) harbours *S. aureus* as a component of the normal skin microbiota, particularly in moist mucosal areas around nostrils and nose mucosa (Balasubramanian et al., 2017). Also, *S. aureus* in healthy humans can colonize skin of axillae, groin, arms, chest and stomach (Otto, 2010). If speaking about niches of *S. aureus* on skin, these niches are sweat glands (Otto, 2010) and hair follicles hollows (Ten Broeke-Smits et al., 2010). Microorganisms on skin form biofilms, and *S. aureus* is not an exception (Ten Broeke-Smits et al., 2010; Jahns et al., 2012; Khorvash et al., 2012; Matard et al., 2013). In some cases of skin disorders like psoriasis, rosacea (Totte et al., 2016) or atopic dermatitis (Hong et al., 2011) an abundant growth and biofilm formation of *S. aureus* occurs (Allen et al., 2014). For example, in case of atopic dermatitis biofilms of *S. aureus* can grow in sweat gland excretion tract, which leads to their occlusion and further inflammatory complications (Allen et al., 2014). It was shown that *S. aureus* is able to form biofilms on the surface of skin wounds which leads to exacerbations, inflammation and regeneration delays (Estes et al., 2011).

Biofilms of *S. aureus* nowadays are an object of intensive research because of the reasons mentioned above. The biofilm matrix of *S. aureus* was deeply studied. It consists of polysaccharides, proteins and extracellular DNA (Lister, Horswill, 2014). QS-system of *S. aureus* was also studied and it is based on autoinducer peptide(s) consisting of thiolactone ring and N-terminal linear part; the receptor is a membrane histidine kinase AgrC which phosphorylates a regulatory protein ArgA (Kim et al., 2017). Signaling pathways of *S. aureus* biofilm formation were investigated pretty in details; sigma-factor B, additional regulator of staphylococci SarA, regulator MgrA and other molecules are involved in their functioning. Biofilms of *S. aureus* and their structures were also studied (van de Belt et al., 2001; Archer et al., 2011).

Because *S. aureus* is a cause of nosocomial infections and a range of other diseases, and in addition because it is a human skin inhabitant, *S. aureus* undergo frequently the action of antibiotics. This over-exposure to antibiotics was leading to the appearance of multiple resistances. Methicillin-resistant *S. aureus* (MRSA) resistant to beta-lactams because of presence of lactamases, represent a major threat for humanity (Green et al., 2012). The number of annual infections caused by MRSA in USA in the middle of 2000-s was about 100000, and the death rate was about 18000 per year, which made MRSA the top of the heap infectious agent, higher than AIDS and tuberculosis (Green et al., 2012). Cases of *S. aureus* resistance to levofloxacin, tetracycline, chloramphenicol, cefoxitin, ciprofloxacin, gentamicin, tetracycline and sulfamethoxazole-trimethoprim were described (Akanbi et al., 2017). One of the resistance mechanisms are multidrug resistance efflux pumps, like Nor, Sdr, TetA and Tet38 (tetracycline efflux pumps), LmrS, Qac and other (Andersen et al., 2015). It was shown in *S. aureus*, that antibiotics in sub-MIC concentrations

stimulate the virulence and biofilm growth. For example, beta-lactams increase the hemolytic activity of *S. aureus* via enhancement of the expression of a two-component system, SaeRS (Kuroda et al., 2007), which is responsible for the synthesis of fibronectin-binding proteins, nucleases, coagulases and hemolysins (Kuroda et al., 2007). In some strains of MRSA, ceftaroline (5<sup>th</sup> generation cephalosporine) at sub-MIC concentrations stimulates the biofilm growth. This process was attributed to an increase of *agrA* (virulence regulating transcription factor, QS) and *icaA* (poly-N-acetylglucosamine or polysaccharide intercellular adhesion (PIA) synthesis) genes expression. However, a correlation between expression of these genes and biofilm growth stimulation was not observed in all cases (Lazaro-Diez et al., 2016). In some MRSA strains, *agr* genes expression is also increased by sub-MIC concentrations of clindamycin and tetracycline (both suppress protein synthesis), which leads to exacerbations of MRSA-infections (Joo et al., 2010). Clindamycin in sub-MIC concentrations also enhances the expression of a range of genes of biofilm formation processes. Those genes encode fibronectin-binding proteins (Fnb), phenol-soluble modulins (*psm*), hydrolases of murein (AltA) and other (Schilcher et al., 2016). A stimulatory effect on biofilm growth was also shown in some MRSA strains after exposure to vancomycin and azithromycin at sub-MIC concentrations (Majidpour et al., 2017). However, MRSA form a very heterogeneous group and in some strains sub-MIC concentrations of azithromycin inhibit  $\alpha$ -hemolysin synthesis and biofilm growth in general (Gui et al., 2014). This antibiofilm effect can be increased by combination with some additives, for example, like acrylic polymer DuraSite® (Wu et al., 2010). Such opposite data reveal the complexity of the biofilm formation processes and support deeper investigations of antibiotic impacts on *S. aureus* and in particular on MRSA.

As in the case of *P. aeruginosa*, in *S. aureus* the QS systems seem to be essential in biofilm resistance to antibiotics and in interaction with them. Besides the *agr*-system, *S. aureus* possesses also a RAP/TRAP system, which affects the *agr* operon and also regulates cell wall synthesis (Brackman et al., 2016). When the RAP/TRAP system is inhibited, for example by hamamelitannine (2,5-di-O-halloyl-D-hamamelose) which interacts with the TraP receptor, the synthesis of the cell wall is indirectly increased, and consequently the extracellular DNA amount is decreased. This is leading to an increase of *S. aureus* biofilms sensitivity to some antibiotics (for instance to vancomycin), because extracellular DNA is an important factor of antibiotic resistance (Brackman et al., 2016). Savirin (*S. aureus* virulence inhibitor) or 3-(4-propane-2-ylphenyl)-sulfonyl-1H-triazolo-(1,5-a)-quinazoline-5-one is a recently found compound which significantly inhibits the virulence of *S. aureus* and facilitates immune system fighting against *S. aureus* through inhibition of the *agr*-system.

Some works are dedicated to investigating the effects of cosmetic components on *S. aureus* biofilms. For example, extract of immature fruits of *Juglans regia* (component of hair dyes) inhibits

the adhesion and biofilm formation of *S. aureus* (Quave et al., 2008). Extracts of leaves of *Castanea sativa* which contains pentacyclic triterpenes, inhibit the biofilm growth of *S. aureus* via interruption of its *agr*-dependent QS-system. The natural surfactant glycerol monolaurate, frequently used in cosmetics, also inhibits the growth of *S. aureus* biofilms (Schlievert & Peterson, 2012; Hess et al., 2015). At sub-MIC concentrations, eugenol (4-allyl-2-methoxyphenol), a component of clove oil used in cosmetics, suppresses biofilm formation by *S. aureus* and inhibits the expression of genes responsible for biofilm assembly. Also, it prevents the colonization of mucosa by *S. aureus* cells *in vivo* (Yadav et al., 2015). At MIC and higher, eugenol accelerates mature biofilm dispersal, causes cell deformation, destruction of cell envelope and cell contents outflow (Yadav et al., 2015). Combination of eugenol with carvacrol (an oregano component) possesses a synergistic effect (Yadav et al., 2015). Carvacrol itself shows antimicrobial and antibiofilm effect on *S. aureus* (Knowles et al., 2005). In binary biofilms of *S. aureus* and *Salmonella enterica* ser. *typhimurium* on initial stages of biofilm formation carvacrol inhibits more efficiently *S. aureus*, and in mature biofilms of *S. enterica* ser. *typhimurium*, extracellular polysaccharides of *S. aureus* matrix play a key protective role (Knowles et al., 2005). Cinnamon bark oil which includes also phenolic compounds, shows antibacterial activity against *S. aureus* and some other microorganisms (Nabavi et al., 2015). Vetiver (*Chrysopogon zizanioides*) essential oil is widely used in perfumery and shows antibacterial and antibiofilm effects on *S. aureus* (Burger et al., 2017). Results of investigation in the framework of present thesis have shown that TWU™ and PS291® inhibit significantly biofilm growth of *S. aureus* without toxic effect - probably via cell surface adhesion and co-adhesion interruption (Gannesen et al., 2018).

Tween 80 is widely used in laboratory experiments, and sometimes in cosmetics as emulsifying, stimulates the growth of biofilms and planktonic cultures of *S. aureus*. The effect is probably based on its surface-active properties: in presence of Tween 80 cell membranes become more permeable, and nutrients and ions – more accessible for bacteria (Nielsen et al., 2016). However, the data concerning Tween 80 are controversial: a work reports that 0.01% of Tween 80 in the medium inhibits the growth of *S. aureus* and *P. aeruginosa* (Toutain-Kidd et al., 2009). This underlines the necessity of additional deeper investigation of Tween 80 effects. Nevertheless, the effects of Tween 80 should be strain-specific. The biocide triclosan is used sometimes in cosmetics and, in sub-MIC concentrations, it stimulates small colonies variant (SCV) phenotype in *S. aureus* (Forbes et al., 2015). Triclosan-mediated SCV is characterized by a weak biofilm formation activity, lower virulence and colony pigmentation, triclosan resistance and increased sensitivity to some antibiotics – tetracycline, kanamycin, ampicillin etc. (Forbes et al., 2015). Cosmetics components can be not only inhibitors of *S. aureus* signalling pathways and biofilm formation, but they can also modify the bacterial metabolism without visible lethal effect. For instance, in *S. aureus* *Glycyrrhiza*

*glabra* root extracts inhibit the synthesis of diacetyl – the main factor of unpleasant sweat odor (Hara et al., 2014). *G. glabra* root extract partially inhibits the membrane lactate dehydrogenase (LDH), thus it interrupts pyruvate synthesis which is essential for the following reactions of acetone and diacetyl synthesis. Associated to *G. glabra* extracts,  $\alpha$ -tocopheryl-l-ascorbate-2-O-phosphate diester potassium salt or EPC-1K also partially inhibits the LDH activity and it partially disturbs the fumarate dehydrogenase complex and malonil-KOA-synthase (Hara et al., 2014).

A number of published works are dedicated to the study of the interaction of human endogenous molecular factors with *S. aureus*. *S. aureus*, similarly to other mentioned skin microorganisms, interacts with different active compounds. For instance, defensins and cathelicidins show direct antibacterial effect on *S. aureus* (Brown et al., 2013). Here active compounds without direct antibacterial activity are described. Already at the end of the last century it was shown that colonization of nostrils mucosa by *S. aureus* correlates in women with estrogen level: the higher cariopicnotic index (and consequently estrogen level) resulted in higher amount of women-carriers of *S. aureus* (Winkler et al., 1990). Later it was shown that the use of hormonal contraceptives based on estrogens and progestins also leads to an increase of *S. aureus* carriage on nose mucosa in women (Zanger et al., 2012). This is supporting the idea of a positive effect of estrogens on mucosa colonization by *S. aureus*. Epinephrine was shown stimulating the growth of *S. aureus* in a model of infected skin wound in swine (Stratford et al., 2002). Catecholamines (epinephrine, norepinephrine) can substitute lacking ligands and iron carriers in mutants of *S. aureus* strains lacking staphyloferrin (staphylococcal siderophore, Beasley et al., 2011).  $\alpha$ -melanocyte stimulating hormone shows antimicrobial and antibiofilm action on *S. aureus* including MRSA *in vitro* (Madhuri et al., 2009). Substance P (neuropeptide which is realized by nerve endings in skin, Severini et al., 2002) increases synthesis of staphylococcal enterotoxin C2 and increases the ability of *S. aureus* to adhere to keratinocytes: the target of substance P in *S. aureus* is the thermo unstable ribosomal elongation factor Ef-Tu (N'Diaye et al., 2016). Earlier it was shown that substance P and also neuropeptide Y do not have any antibacterial activities against *S. aureus* (Hansen et al., 2006; Karim et al., 2008), but these compounds and also calcitonin-gene related peptide possess antibacterial activity against other microorganisms – *P. aeruginosa*, *Streptococcus mutans*, *Candida albicans* (Karim et al., 2008). Corticosteroids inhibit the growth of *S. aureus*. For instance, a combination of the antibiotic mupirocin and hydrocortisone butyrate efficiently decreases the amount of *S. aureus* on skin areas affected by eczema and atopic dermatitis (Gong et al., 2006). *In vitro* some synthetic corticosteroids (fluticasone, mometasone, budesonide) shows antibacterial and antibiofilm activity against *S. aureus* (Goggin et al., 2013).

In present work it was shown that human NUPs affect significantly the biofilm formation activity of *S. aureus* and *S. epidermidis* (Gannesen et al., 2018). The effect of NUPs depend on

cultivation conditions: at 37°C the growth of monospecies *S. epidermidis* biofilms is stimulated and in *S. aureus* – inhibited (it results in changes of thickness and biomass density of biofilms). At 33°C the effect of NUPs on *S. epidermidis* biofilms is reversed. In binary biofilms at 37°C NUPs are able to increase the competitive ability of *S. epidermidis* against *S. aureus*. The effects of NUPs appears mainly in case of biofilms and less in planktonic cultures, this allows us to suggest a regulatory function of NUPs in interactions between human organism and its microbiota (Gannesen et al., 2018).

### 1.3.5. *Staphylococcus epidermidis*

*S. epidermidis* is another representative of the *Staphylococcus* genus. It is one of the most abundant microorganisms of human skin and prefers moist and sebaceous skin areas – axillae, head, mucosa (Otto, 2009). *S. epidermidis* is closely interconnected with the human physiology and his skin. To date it is widely thought that *S. epidermidis* is the first microorganism which a fetus meets in utero. This fact is approved by the abundant presence of *S. epidermidis* in amniotic liquid and meconium (Bresco et al., 2017). Also, *S. epidermidis* is one of the first microorganisms which colonizes the skin of a newborn (Bresco et al., 2017). *S. epidermidis* lacks coagulases and thus belongs to the group of coagulase-negative staphylococci (Otto, 2009). *S. epidermidis* has a range of advantageous features on human skin. For instance, *S. epidermidis* synthesizes compounds which increase the ability of keratinocytes to withstand *S. aureus* and group A streptococci by  $\beta$ -defensins synthesis. The mechanism of  $\beta$ -defensins is based on interaction of lipopeptides of *S. epidermidis* with TLR2-receptors, which launch the cascade of reactions (Lai et al., 2010). Also it was shown that *S. epidermidis* in skin synthesizes 6-N-hydroxyaminopurin. This compound inhibits the growth of tumors generated by exposition to ultraviolet irradiation (Nakatsuji et al., 2018). *S. epidermidis* is excreting some compounds with antibacterial activity. It is shown that *S. epidermidis* can decrease the metabolic activity and expression of a number of genes in *S. aureus* when microbes are in dual-species biofilms (Vandecandelaere et al., 2017). This suggests the potential antibacterial activity of *S. epidermidis*. Delta-lysin E229DL produced by *S. epidermidis* is low-toxic for humans and highly active against *S. aureus* (Al-Mahrous et al., 2010). The peptide 487 synthesized by *S. epidermidis* (and encoded by the *hlp* gene) is showing sequence homology with hemolysins, but it is less toxic and possesses antibacterial activity (Al-Mahrous et al., 2011). A range of lantibiotics is produced by *S. epidermidis* – Pep5, epilancine K7 and epicidin 280, which inhibit the growth of MRSA and some other coagulase-negative staphylococci associated with human diseases (Christensen, Bruggemann, 2014). The antimicrobial activity of *S. epidermidis* also appears in its feature to produce short-chain fatty acids and especially succinate, which inhibits effectively the growth of *Cutibacterium acnes* and decreases inflammation caused by *C. acnes* in skin ulcers *in vivo* (Wang et al., 2014). This allows to consider *S. epidermidis* as a potential skin

probiotic. Also, *S. epidermidis* produces glycerol, which maintains optimal moisture of the skin, subacid pH-level and improves skin texture (Nodake et al., 2015). Moreover, *S. epidermidis* superoxide dismutases possesses a protective activity against reactive oxygen species which is also favorable for the skin (Nodake et al., 2015). *S. epidermidis* stimulates the synthesis of NO in skin. NO is a compound which facilitates human cells defense against infections (Carey et al., 2016). This stimulation was attributed to the production of small molecules of non-peptide nature synthesized by *S. epidermidis*. It is interesting to note that *S. aureus* has a similar effect (Carey et al., 2016).

However, despite a number of useful properties, *S. epidermidis* is considered as an opportunistic pathogen causing a number of diseases and hospital infections, although not as dangerous as *S. aureus* (Otto et al., 2009). Most of the diseases caused by *S. epidermidis* occur in the case of infection of artificial implants after insufficient sterility of surgical operations. *S. epidermidis* forms biofilms on the surface of artificial implants, which leads to the treatment and infections of venous catheters, bone implants, artificial heart valves (Schommer et al., 2011), and biofilm formation is the first of the two main causes of danger of *S. epidermidis* (Saffari et al., 2016). From 50 to 70% of all infections linked to artificial central nervous system implants are caused by *S. epidermidis* (Schaeffer et al., 2015), and some researchers designate *S. epidermidis* as the key pathogen of such infections (Wang et al., 2010). In general, up to 20% of all cases of infection on artificial implants occur because of *S. epidermidis*, and *S. epidermidis* appears responsible for 50% of infections on implants delayed in time (Bresco et al., 2017). Speaking about the skin, *S. epidermidis* can also grow abundantly on the skin areas affected by atopic dermatitis: its incidence is about 30-40%, while in other cases, atopic dermatitis is dominated by *S. aureus* (Hon et al., 2016). The danger of *S. epidermidis* is the formation of biofilms, which, like in the case of other microorganisms described earlier, are resistant to antimicrobial compounds and seriously hamper treatment. Polysaccharide extracellular adhesin (as in *S. aureus*) and extracellular proteins Embp and Aap - the adhesins responsible for the attachment of cells to the surface and to each other (Schommer et al., 2011) play a key role in the formation of *S. epidermidis* biofilms, in particular on the implant surface.. Also, at the initial stages of adhesion, the Bhp protein (homologous protein to Bap) and the autolysin-adhesin AltE play the significant role (Salgueiro et al., 2017). In the biofilm, *S. epidermidis* is also more resistant to the human immune system: *S. epidermidis* biofilms cause a lower intensity of interleukin 1 $\beta$  synthesis, less activation of NF- $\kappa$ B and less macrophage activity than *S. epidermidis* strains unable to form well-developed biofilms (Schommer et al., 2011). *S. epidermidis* virulence factors are close to that expressed by *S. aureus* and they can act on a multitude of targets. They include extracellular adhesins (polysaccharides, proteins attached to elastins and fibronectin), toxins (pro-inflammatory cytolytins), extracellular proteases, lipases, lytic



enzymes and antimicrobial peptide (Otto, 2012). Also, *S. epidermidis* forms poly- $\gamma$ -glutamic acid, a virulence factor that inhibits the synthesis of antimicrobial peptides and phagocytosis (Kleinschmidt et al., 2015). Synthesis of many virulence factors, as shown by clinical studies, is associated with the growth of *S. epidermidis* in the form of biofilms during implant-associated infections. For example, phenol-soluble modulins synthesized by *S. epidermidis* in biofilms on implants can cause neutrophil lysis, which also complicates the course of the disease (Cheung et al., 2010). Lipoteichoic acid of *S. epidermidis* stimulates the release of tumor necrosis factors and interleukin 1-compounds that induce hemodynamic shock and tissue damage in bacteremias and sepsis (Wakabayashi et al., 1991).

The consequence of infections of implants with *S. epidermidis* biofilms may be bacteremia, which is difficult to treat. If we talk about the effect of antibiotics on *S. epidermidis*, then according to some estimates up to 80% of *S. epidermidis* strains forming biofilms on implantable devices are multi-resistant (Kleinschmidt et al., 2015). The reduced sensitivity of *S. epidermidis* to antibiotics, which is increasingly encountered in clinical practice, is the second major cause of the risk of *S. epidermidis* for human health (Saffari et al., 2016). One of the most common multiresistant genotypes, *S. epidermidis* ST2, was isolated from the bloodstream of patients with sepsis, knee implants, intervertebral disc tissue (Widerstrom et al., 2012), and tracheal tubes (Saffari et al., 2016). According to the results of the analysis, the ST2 genotype is a methicillin-resistant *S. epidermidis* (resistance is due to the presence of penicillin-binding proteins) but this strain is also resistant to oxycillin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, mupirocin, gentamicin and trimetopimosulfomethoxazole (Lorio et al. 2012). Especially dangerous are ST strains (not only ST2, but also others) that acquire the so-called “heterostability to vancomycin” an antibiotic widely used in the treatment of infections caused by MRSA and multidrug-resistant *S. epidermidis* (MRSE, van Hal, Fowler, 2013). Heterostability to an antibiotic is a phenomenon in which cells of a microorganism from a single subpopulation that seems to be the same in properties but exhibits enhanced resistance to the antibiotic (El-Halfawy, Valvano, 2015). Vancomycin-heteroresistant MRSE is currently considered one of the main pathogens of blood flow infections, especially dangerous for premature infants in intensive care (Chong et al., 2016). There is evidence for the existence of azithromycin-resistant strains of *S. epidermidis*, and studies show that the combination of azithromycin with other antibiotics inhibits the growth of bacteria, but does not affect the biofilms formed (Presterl et al., 2009).

It has been shown that subinhibitory concentrations of a number of antibiotics can stimulate the growth of *S. epidermidis* biofilms. Tigecycline and ciprofloxacin increase the expression of the *icaA*, *altE*, *sigB* (regulatory sigma-B) coding factors of the biofilm phenotype (Szczyka et al., 2017). Erythromycin, tetracycline and synthetic streptogramin in subinhibitory concentrations

increase the expression of the *ica* operon and the formation of *S. epidermidis* biofilms (Rachid et al., 2000). Vancomycin in subinhibitory concentrations weakens the growth of *S. epidermidis* biofilms, however, the concentration of extracellular DNA in the matrix increases of tens times in the biofilm. This can increase the stability of *S. epidermidis* biofilms in regard to vancomycin since extracellular DNA is a molecular trap for the antibiotic (Doroshenko et al., 2014). Azithromycin in subinhibitory concentrations may also stimulate the growth of *S. epidermidis* biofilms in strains possessing the *icaABCD* operon and non-mutant strains thereon (Wang et al., 2010). However, in strains containing the wild operon *icaABCD*, the stimulation is more effective (Wang et al., 2010) suggesting several key processes as the "targets" of the antibiotic into the cell. An interesting work from Eroshenko showed an inhibitory effect of N-acetylglucosamine on the growth of *S. epidermidis* biofilms (Eroshenko et al., 2017) whereas subinhibitory concentrations stimulate the growth. Interestingly, a mutant strain incapable of synthesizing polysaccharide extracellular adhesin is more resistant to the action of N-acetylglucosamine, since this compound has a mucolytic effect (Eroshenko et al., 2017).

Speaking about the interaction of cosmetics with *S. epidermidis*, it should be noticed that this microorganism is a producer of diacetyl along with *S. aureus*, contributing to the unpleasant odor of sweat (Hara et al., 2014). Similarly, as in the case of *S. aureus*, the synthesis of diacetyl is efficiently inhibited by licorice root extract and EPC-1Kb as already mentioned (see section 1.2.3.4., Hara et al., 2014). It was shown that extracts from a number of plants from the dry forests of the Kaatinga in Brazil have an antimicrobial and antibiotic effect on *S. epidermidis* (Trentin et al., 2011). The berberine alkaloid, isolated from the Coptic chrysanthemum (*Coptidis rhizoma*) and barberry (*Berberis vulgaris*) and potentially effective as a component of cosmetics (Lee et al., 2018), has an antimicrobial and antibiofilm effect on *S. epidermidis* (Wang et al., 2009). It has also been shown that berberine disrupts the adhesion of *S. epidermidis* to titanium surfaces, which makes it a potential agent for implant treatment, because berberine is safe for humans in low concentrations (Wang et al., 2009a). The extract of mint (*Mentha pulegium*), consisting mainly of piperitone, piperitenone,  $\alpha$ -terpineol and pulegone, substances used in perfumery, has a strong antimicrobial effect on *S. epidermidis* and *S. aureus* (Mahboubi, Haghi, 2008). Essential oil of oregano (oregano - *Origanum vulgare*), including carvacrol and thymol - compounds used in perfumery, also has a strong antibacterial and anti-biofilm effect on *S. epidermidis* and *S. aureus*, however, biofilms are several times more resistant to the inhibitory effects than planktonic cells (Nostro et al., 2007). Oregano is effective against both methicillin-sensitive strains and against methicillin-resistant strains (Nostro et al., 2004). An anti-biofilm effect on *S. epidermidis* is also exerted by Tween 80 (Toutain-Kidd et al., 2009). Low viscosity chitosan, as used in cosmetology, has an antimicrobial and anti-biofilm effect on *S. epidermidis*. Similarly, *S. aureus* and *S.*

*epidermidis* resistant to methicillin, are sensitive to vetiver essential oil (Burger et al., 2017).

A number of works are devoted to the interactions of *S. epidermidis* with the regulatory systems of the human body, however, their number is less than in the case of *S. aureus*. Similarly, let's consider only those compounds that was not previously considered antibacterial. The calcitonin-gene-related peptide (CGRP) increases the adhesion of *S. epidermidis* to keratinocytes, and also increases the ability of *S. epidermidis* to stimulate the synthesis of interleukin 8. The effect of CGRP disappears when the mechanosensitive ion channels are blocked by gadolinium chloride (N'Diaye et al., 2016). The chaperone DnaK was identified as the CGRP receptor in *S. epidermidis* (N'Diaye et al., 2016a). The growth of biofilms of *S. epidermidis* is stimulated by substance P, and like *S. aureus*, the membrane form of the elongation factor Ef-Tu in *S. epidermidis* is the receptor of substance P (N'Diaye et al., 2016). In this case, substance P has a broad spectrum of action: the effect of substance P is shown on Gram-positive and Gram-negative microorganisms (N'Diaye et al., 2016). Inotropic hormones of catecholamine nature stimulate the growth of *S. epidermidis* biofilms and increase the synthesis of matrix exopolysaccharides (Lyte et al., 2003).

#### **1.3.6. *Cutibacterium acnes***

*C. acnes*, formerly belonging to the genus *Propionibacterium* (Scholz, Kilian, 2016) is one of the most abundant microorganisms of the human skin. In some areas of the skin, it is practically the only microorganism isolated. *C. acnes* is closely associated to humans which may be its principal host, and its abundance on the skin may be a consequence of the positive effect of such coexistence (Christensen, Bruggemann, 2014). The positive effect of *C. acnes* can be revealed for instance, in the context breast cancer :it was shown in a mouse model that *C. acnes* in combination with melatonin stimulates tumor necrosis, suppresses excessive angiogenesis and stimulates the Th1-cytokine-mediated antitumor response (Talib, Saleh, 2015). *C. acnes* are gram-positive immobile, facultative anaerobic coryneomorphic rod-shaped bacteria (Aubin et al., 2014), preferring sebaceous and moist skin areas (forehead, neck, back, Grice et al., 2009; Aubin et al., 2014; SanMiguel, Grice, 2015, Feuerstein et al., 2017). Due to the production of lipases, *C. acnes* can consume sebum and it is essentially found inside the hollows of the sebaceous glands and hair follicles (Lee et al., 1982). The genus *Propionibacterium* was recently divided into three genera - *Acidipropionibacterium*, *Cutibacterium*, *Pseudoropionibacterium*, on the basis of the ratio of G-C pairs in the genome, the structure of the peptidic bridges of peptidoglycan, and but also in regard of ecological data and *Cutibacterium acnes* was selected because of its association to the human skin (Scholz, Kilian, 2016). Currently *C. acnes* is considered as an opportunistic human pathogen associated with a variety of diseases. First of all, this concerns a variety of implants infections (shoulder implants, Achermann et al., 2014, cardiac implants, Okuda et al., 2018), contact lenses infections (Perry, Lambert, 2011). *C. acnes* biofilm infections occur frequently in shoulder implants

(Achermann et al., 2014) as *C. acnes* is more common in this skin area. The danger of the infections of internal implants caused by *C. acnes* is that they appear a long time after the operation, even sometimes several years after (Achermann et al., 2014) because of the slow growth of this microorganism. These biofilms on the surface of the implants, complicate strongly the treatment as *C. acnes* can be very hard to eliminate (Achermann et al., 2014).

Speaking of acne vulgaris, many researchers now consider *C. acnes* clearly associated to acne (Fitz-Gibbon et al., 2013; Achermann et al., 2014; Pettersson et al., 2018). However, *C. acnes* is present both on healthy and acneic skin and correlating results from ribosomal RNA genes sequencing, clinical observations, and analysis of the distribution of *C. acnes* strains on the human skin, it appeared that *C. acnes* should be divided into several ribotypes, some being called acneic while other ones should be designated as non-acneic (Fitz-Gibbon et al., 2013). In this regard, some researchers consider that the link between acne and *C. acnes* is not clearly demonstrated (Khorvash et al., 2012; Totte et al., 2016). Nevertheless, there are reasons to believe that *C. acnes* is one of the causes leading to the development of acne. For example, *C. acnes* can modulate the proliferation of keratinocytes and enhance local inflammation, which is important for the formation of acne. Also, because to its ability to consume fats, *C. acnes* is involved in the processes of lipogenesis in the skin and the formation of sebum (Dessinioti, Katsambas, 2016). *C. acnes* interacts with the human immune system: with TOLL-like receptors, antimicrobial peptides, protease receptors and intercellular metalloproteinases; stimulates the synthesis of a number of pro-inflammatory cytokines in white blood cells, keratinocytes and sebocytes (Dessinioti, Katsambas, 2016). It is shown that the expression of TLR-2 and TLR-4 receptors in keratinocytes in acne ulcers increases (Achermann et al., 2014). Also, perhaps, the stimulation of the synthesis of interleukin 1 $\alpha$  *C. acnes* promotes the formation of comedones (Dessinioti, Katsambas, 2016). *C. acnes* also stimulates the synthesis of  $\beta$ -defensins and pro-inflammatory cytokines in sebocytes, which affects the growth and differentiation of sebocytes, as well as the inflammatory process in the foci of acne (Nagy et al., 2006). It was shown that *C. acnes* stimulates the synthesis of the neuropeptide corticotiberin in the epidermis, which also affects the differentiation of keratinocytes and the formation of comedones (Isard et al., 2009). In comedones and inflamed ulcers, *C. acnes* forms biofilms (Ten Broeke-Smits et al., 2010; Khorvash et al., 2012; Jahns et al., 2012; Matard et al., 2013; Jahns, Alexeyev, 2014), which can be one of the causes of acne and complications associated with it. Blockage of pores on the skin and formation lead to the creation of conditions favorable for the development of *C. acnes* and its biofilms (hyperproliferation of keratinocytes, sebum excess, Williams et al., 2012).

*C. acnes* biofilms are currently being studied quite intensively, but they remain an unexplored object. It is known that this is a slowly growing microorganism, and biofilm formation in laboratory experiments requires from 2 days (Chiba et al., 2015) up to 7 days or more (Tyner, Patel, 2016).

There are some data on the composition of the matrix of *C. acnes* biofilms (Jahns et al., 2016, Okuda et al., 2018): it was shown that, as many other biofilms, the matrix of *C. acnes* biofilms contains DNA, proteins and polysaccharides (Okuda et al., 2018). However, this work raises a number of questions, particularly in regard of the methodology employed to obtain the matrix. These questions will be discussed in detail in the chapter "Discussion". *C. acnes* biofilms are now considered as one of the reasons for the stability of *C. acnes* during the antibiotic treatment of acne (Dessinioti, Katsambas, 2016).

Data on the resistance of *C. acnes* to antibiotics are available. Strains collected in patients with acne are often resistant to at least one antibiotics (Dessinioti, Katsambas, 2016). Clinical strains from patients with acne can be resistant to macrolides, streptogramins and lincosamides (Ross et al., 2001). Resistance to erythromycin is often associated with resistance to clindamycin (Ross et al., 2001; Coates et al., 2002). It was also reported the identification of strains resistant to tetracyclines and doxycycline (Coates et al., 2002, Sardana et al., 2016). Resistance to macrolides, streptogramins and lincosamides is due to a mutation in the 23S-rRNA gene, and for tetracyclines to a mutation in the 16S-rRNA gene (Dessinioti, Katsambas, 2016). In general, *C. acnes* resistance to antibiotics is due mainly to different chromosomal mutations (Dessinioti, Katsambas, 2016).

A number of works are devoted to the role of *C. acnes* in the development of acne and studies of various cosmetic compounds that inhibit its growth or virulence. It is shown that the population of *C. acnes* on the face skin is increased in people who use moisturizing cosmetics (cream, emulsion), while the amount of *S. epidermidis* does not change (Lee et al., 2018a). There are data on the inhibitory effect of cinnamon bark extracts on the growth of a number of skin microorganisms, including *C. acnes* and *S. epidermidis* (Nabavi et al., 2015), the anti-inflammatory and inhibitory action of the complex of bakuchiol for *C. acnes* (meroterpen, secreted from *Psoralea corylifolia*), extract of ginkgo biloba and mannitol (Trompezinski et al., 2016). Also, it was found that the precursor of vitamin C - magnesium ascorbyl phosphate - suppresses the process of lipid peroxidation by *C. acnes* (Lee et al., 2016). The conjugate of chitosan and caffeic acid have an antibacterial effect on antibiotic-resistant strains of *C. acnes* and *P. aeruginosa*, while enhancing the action of antibiotics on these strains (Kim et al., 2017a). Polyunsaturated long-chain fatty acids, in particular linoleic and docosahexaenoic acids, which are present in essential cosmetic oils (Rabasco Alvarez, Gonzalez Rodriguez, 2000), have antibacterial action on *C. acnes* and *S. aureus* (Desbois, Lawlor, 2013), which makes them potential agents to fight against infections caused by these microorganisms. Lauric acid, one of the free fatty acids of sebum, has an antibacterial effect on *C. acnes*, consisting in the destruction of membranes. A technique of liposomal delivery of lauric acid to foci of acne has been even developed (Yang et al., 2009). EDTA, being a component of cosmetics, enhances the antibacterial effect of other compounds on *C. acnes* and other

microorganisms, possibly acting as a membrane permeabilizer (Pannu et al., 2011). However, very few papers are devoted to *C. acnes* biofilms, and studies of the effects of cosmetics on the growth of *C. acnes* biofilms are practically not carried out. Given some evidence of the development of *C. acnes* biofilms within the hollows of the sebaceous glands (Jahns et al., 2012), this aspect seems very important and interesting. For example, there is evidence that *Myrtus communis*, used in cosmetics (Ben Hsouna et al., 2014), has an antibacterial and antibiofilm effect on *C. acnes*, which makes it a potential agent against acne (Feuilloley et al., 2015). *Rosmarinus officinalis* extract has a multifactorial effect: first, it inhibits the growth of *C. acnes* biofilms and inhibits cell adhesion; Secondly, rosemary extract reduces the synthesis of tumor necrosis factor and pro-inflammatory interleukins in eukaryotic cells (Tsai et al., 2013). An interesting work was devoted to the effect of N-acetylcysteine on the growth of cultures and biofilms of *C. acnes* and *S. epidermidis* (Eroshenko et al., 2017). The authors showed that N-acetylcysteine, which breaks disulfide bridges between polysaccharide molecules, strongly suppresses the growth of *C. acnes* biofilms.

As a result of the research within the framework of this dissertation, it was found that Uriage<sup>TM</sup> thermal water and PS291®, an oligosaccharide rich in rhamnose, have a strong antibiofilm effect on acneic strains of *C. acnes* without significant cytotoxic effect on keratinocytes. Presumably, the mechanism of action of the compounds is an inhibition of the adhesion of cells to the surface (Gannesen et al., 2018).

Regarding the effect of regulatory human molecules, not previously considered antibacterial, there are few data in *C. acnes* in the literature at present. There is evidence that epinephrine and norepinephrine can both inhibit or stimulate the growth of *C. acnes*, depending on the strain (Boyanova, 2017). In this case, norepinephrine stimulates the disintegration of *C. acnes* biofilms inside atherosclerotic plaques in the arteries, abundant release of proteases, lipases and transferrin, which can potentially complicate the course of the disease (Boyanova et al., 2017).

#### **1.4. Binary biofilms of some microorganisms of human skin**

Since microorganisms exist naturally in the form of multi-species biofilms, such as, for example, dental plaques (Nikolaev & Plakunov, 2007) or in anammox communities in wastewater treatment reactors (Nozhevnikova et al., 2015), the transition from the study of mono-species cultures and biofilms to multi-species ones is actual. However, till now in the scientific literature and laboratory practice mono-species models dominate, and it's only recently (although some works appear at the beginning of the 2000s), that the interest in the research of multi-species biofilms of microorganisms and interactions of members of these communities with each other, as well as the influence a variety of active compounds on these biofilms consisting of several types of microorganisms, started to arise. The simplest multi-species system is binary biofilm. An analysis of the literature at the moment shows that microorganisms in binary biofilms constructed in the

framework of the experiment do not remain inert and actively influence each other. For example, the work of Zhurina et al., devoted to reconstructed biofilms of microorganisms of stratal waters in oil fields, shows that in the presence of halophile on a halophilic medium, a halotolerant microorganism survives better and consumes oil products more efficiently than without an halophil partner (Zhurina et al., 2008). In this case, the mechanism of protection is the consumption of ectoin and glutamate isolated by *Chromohalobacter* sp. by the halotolerant microorganism *Dietzia* sp. Ectoin and glutamate are concentrated in the biofilm and do not dissipate into the medium because of the presence of the matrix (Zhurina et al., 2008). The presence of *Enterococcus faecalis* in a binary biofilm with *P. aeruginosa* stimulates the synthesis of polysaccharides of the Psl and Pel matrix, which make the binary biofilm thicker, and probably contributes to complications in infections and a better protection of microorganisms within the biofilm. These microorganisms are often released together when sampling patients (Lee et al., 2017). Expression of a large number of genes varies in *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* in a binary biofilm compared with mono-species: in *S. mutans*, the expression of the alternative sigma factor SigX regulating the synthesis of bacteriocins and cellular competence (Szafrński et al., 2017) increases, whereas *A. actinomycetemcomitans* increases the synthesis of a number of enzymes, including chinol peroxidase, nucleotide metabolism enzymes, and others (Szafrński et al., 2017). Both microorganisms are components of dental plaques, with *S. mutans* being a model cariogenic microorganism (Szafrński et al., 2017). A similar enhancement of SigX expression in *S. mutans* occurs in binary biofilms with *Candida albicans*, while it suppresses matrix synthesis, however, the number of cells and biomass of binary biofilms increase (Sztajer et al., 2014). The presence of *Listeria monocytogenes* in a binary biofilm with *P. putida* increases the resistance of the *P. putida* to the antiseptic benzalkonium chloride, with no adaptation to benzalkonium chloride observed in the experiment (Giaouris et al., 2013). *S. mutans* stimulates the adhesion of cells and the growth of *Streptococcus sanguinis* in binary biofilm in comparison with mono-species; both microorganisms are cariogenic agents of dental plaques (Shafiei et al., 2016). In this case, nicotine stimulates the growth and synthesis of the polysaccharides of the *S. mutans* matrix in a binary biofilm with *S. sanguinis* (Li et al., 2014), which may be one of the causes of the increased risk of caries in smokers. It is shown that *E. coli* actively colonizes *Ralstonia insidiosa* biofilms under conditions of a lack of nutrients. *R. insidiosa* is a Gram-negative microorganism that lives predominantly in a variety of water systems (water intake stations, etc.) and has the ability to form very powerful biofilms that protect *E. coli* from adverse factors (Liu et al., 2015).

If we consider the binary biofilms of skin microorganisms, here, first of all, it is worth to note the interaction of staphylococci and fungi of the genus *Candida* as the most frequently studied. The positive interaction of the yeast *C. albicans* and *S. aureus* in binary biofilms was shown. Moreover,

*S. aureus* grows several times better in different types of binary biofilms, whereas *C. albicans* can grow worse if *S. aureus* cells were previously adhered on the surface, *i.e.* there is competition at the stage of adhesion (Budzyńska et al., 2017). The importance of extracellular DNA involved in the organization of the three-dimensional structure of binary biofilms was demonstrated (Kean et al., 2017). It has been shown that the stimulating growth of *S. aureus* is exerted by prostaglandin E2, excreted by *C. albicans* (Krause et al., 2015). *C. albicans* does not only improves the growth of *S. aureus* in binary biofilms, but also protects it against biocides, such as for example, vancomycin (Kong et al., 2016) and miconazole (Kean et al., 2017). Also,  $\beta$ -1,3-glucan, which is a component of *C. albicans* cell walls, has a stimulatory effect on *S. aureus* and increases its resistance to vancomycin in binary biofilms (Kong et al., 2016). Farnesol, released during the metabolism of *C. albicans*, may stimulate the pathogenic properties of *S. aureus* through induction of an oxidative stress and the subsequent activation of a number of cellular processes (Kong et al., 2017).

A number of works are devoted to the study of binary biofilms of *S. epidermidis* with fungi of the genus *Candida*. Tan et al. with references to some works, argue that the combination of fungi of the genus *Candida* and bacteria in biofilm is the most common (Tan et al., 2017), which, however, is a controversial statement. Analysis of the structure of binary biofilms of *C. albicans* and *S. epidermidis* showed that in mature biofilms, *C. albicans* forms predominantly hyphae, to which clusters of *S. epidermidis* cells attach abundantly (Chen et al., 2015). The biofilm matrix of *S. epidermidis* has been shown to protect *C. albicans* cells from the action of the fungicide fluconazole (Adam et al., 2002). The *C. albicans* matrix, in turn, protects *S. epidermidis* cells from the action of vancomycin (Adam et al., 2002). In general, *S. epidermidis*, like *S. aureus*, grows better in binary biofilms with *C. albicans* than in mono-species, similarly to *C. albicans*, and one of the reasons of this symbiosis is the expression of the extracellular adhesin *icaA*, a fibronectin-binding protein *fbp* and a surface protein *aap* (Wang et al., 2015). Aap is an adhesion protein that forms rod-shaped fimbriae on the cell surface that participates in the adhesion of cells to the surface of epithelial cells and to each other and provides one of the adhesion mechanisms independent of the polysaccharide cell-cell adhesin (Schaeffer et al., 2015). Another reason for the improvement of *C. albicans* and *S. epidermidis* binary biofilms compared to mono-species biofilms is extracellular DNA from *S. epidermidis* cells (Pammi et al., 2013). In binary biofilms, the expression of *S. epidermidis* genes changes: 2.7% of *S. epidermidis* genes were upregulated and 6% were down regulated, among down regulated genes the *lgr* operon of autolysis of cells repressor presents. The expression of *lrgA* and *lrgB* decreases of 27 and 36 times, respectively (Pammi et al., 2013). Against the binary biofilms of *C. albicans* and *S. epidermidis*, carboxymethyl chitosan is effective, which 5-fold reduces the fouling of silicone plates, making this compound a promising agent for the treatment of vocal prostheses (Tan et al., 2016).



The work of Tyner & Patel is the only article devoted to the binary biofilms of *S. aureus* and *C. acnes* at the time of writing of this thesis. It shows that *C. acnes* biofilms at 37 ° C allowed the viability of *S. aureus* cells to remain longer under anaerobic conditions, while the amount of CFU of *C. acnes* in the presence of *S. aureus* did not change (Tyner& Paterl., 2016). The work of Eroshenko et al., the only article at the time of writing the dissertation devoted to partially binary biofilms of *S. epidermidis* and *C. acnes*, shows that N-acetylcysteine inhibits the growth of mono- and binary biofilms of *C. acnes* and *S. epidermidis*, with *C. acnes* being more sensitive to the action of the drug, rather than *S. epidermidis*. The authors believe that, by this way, N-acetylcysteine can regulate the composition of the microbiota on the skin, and presume that a long-term use of the drug should modulate the balance of the microorganisms. Interestingly, at a low concentration (approximately 1/20 MIC), N-acetylcysteine stimulates the growth of a binary biofilms, presumably through stimulation of *S. epidermidis* growth (Eroshenko et al., 2017). However, the antibacterial effect of N-acetylcysteine remains a subject of debate, since Yin et al. demonstrated a stimulating effect of the compound on the growth of biofilms of *S. aureus*, *S. epidermidis*, *E. faecalis*, *P. aeruginosa*, *A.baumannii* and *K. pneumoniae* (Yin et al., 2018). Perhaps, there is a strain specificity here. In the binary biofilms of *S. aureus* and *S. epidermidis*, both microorganisms change the expression level of a number of genes, and the metabolic activity of *S. aureus* decreased in the presence of *S. epidermidis*, while in *S. epidermidis* the expression of resistance genes to a number of antibiotics was increased (Vandecandelaere et al. 2017). In the course of this dissertation, it was shown that the interaction of *S. aureus* and *S. epidermidis* in a binary biofilm appears to depend on the strain, since *S. aureus* MFP03 has a significant competitive advantage over *S. epidermidis* MFP04 (Gannesen et al. 2018) in both planktonic culture and biofilm. Both strains are isolated from the skin of healthy volunteers (Hillion et al., 2013). In this case, natriuretic peptides of A and C types change the balance of microorganisms: the proportion of *S. epidermidis* in binary biofilm increases with respect to control without additions (Gannesen et al., 2018).

In biofilms of staphylococci with gram-negative microorganisms, the situation is different. For example, Makovcova et al. reported a suppression of *S. aureus* growth in binary biofilms with *E. coli* and *Salmonella enterica* compared to mono-species biofilms, while both Gram-negative microorganisms grew better in the presence of *S. aureus* (Makovcova et al., 2017). Also, binary biofilms of *S. aureus* and *Salmonella* sp. are less resistant to disinfectants (peracetic acid, cetrimonium bromide) than mono-species biofilms. In both microorganisms, the ability to adhere to the surface of polypropylene B (Iñiguez-Moreno et al., 2018) was enhanced in binary biofilm. In the case of binary biofilms *P. aeruginosa* and *S. aureus* *in vitro*, aimed at reproducing the *in vivo* situation in infections (they are often isolated simultaneously in the sampling of patients with cystic fibrosis, otitis, Yadav et al., 2017), *S. aureus* is significantly suppressed by *P. aeruginosa*: 100 cells

of *P. aeruginosa* account for only one cell of *S. aureus* (Miller et al., 2017). This can be explained by phenazines synthesis by *P. aeruginosa* which can exert antimicrobial activities, including on *S. aureus* and *S. epidermidis* (Borrero et al., 2014). While the expression of about 5% of the genome of *S. aureus* changes (virulence increases and metabolic activity decreases) the expression of only 0.3% of the genes is altered in *P. aeruginosa* (Miller et al., 2017). *In vivo* and *in vitro* studies showed some inhibition of MRSA development in the presence of *P. aeruginosa* using *in vitro* systems, but no similar suppression occurs in the *in vivo* model of otitis in rats (Yadav et al., 2017). In this case, both bacteria in binary biofilms express genes that are not expressed in mono-species biofilms: virulence genes, interactions with the immune system, interactions with the host's nervous system, inflammatory factors, etc. (Yadav et al., 2017). However, pseudomonads not only suppress the growth of *S. aureus*, and even protect it from external antibiotics. The *P. aeruginosa* Psl polysaccharide plays an important role in protecting not only the *P. aeruginosa* themselves from antibiotics, but also neighbor microorganisms that are unable to synthesize it or even acquiring the Psl gene by transfer. As a result, *S. aureus* in a binary biofilm with *P. aeruginosa* is less sensitive to tobramycin (Billings et al., 2013). There is evidence that extracellular compounds synthesized by *P. aeruginosa* are able to protect *S. aureus* from the action of antibiotics in biofilms and in planktonic cultures. Thus, *S. aureus* is more resistant to gentamicin in the presence of *P. aeruginosa*, possibly because of the synthesis of aminoglycoside-modifying enzymes by *P. aeruginosa* that protect both bacteria (DeLeon et al., 2014). Also, *P. aeruginosa* synthesizes a 4-hydroxy-2-heptyl-quinoline-N-oxide that blocks the respiration in *S. aureus* and thus protects it from tobramycin (DeLeon et al., 2014).

The presence and appearance of the second microorganism in the binary biofilm influences the interaction of *S. aureus* in the composition of binary biofilms with phages: different microorganisms (lactic acid bacteria, *Enterococcus faecium*) had different effects on the yield of phage particles after seeding on binary biofilm (Gonzalez et al. 2017). Interestingly, in the *S. aureus* binary biofilm with *Lactobacillus plantarum*, after treatment with phages, the amount of CFU of *L. plantarum* increased several fold as compared to the untreated control, indicating a regulatory function of phages in multi-species communities of microorganisms. It was shown *in vitro* that *S. aureus* suppresses the growth of *Aspergillus fumigatus* in binary biofilm, destroys the structure of hyphae and slows down their growth (Ramírez Granillo et al., 2015). Phages from the family *Myoviridae* are active against mono- and binary biofilms of *S. aureus* and *S. epidermidis* (Gutiérrez et al., 2015).

A number of works are devoted to binary biofilms of *M. luteus* with other microorganisms. For example, *Sphingomonas natatoria*, a freshwater gram-negative microorganism (Min, Ricard, 2009), forms dense biofilms on glass if previously it was covered with a layer of *M. luteus* cells. In

their absence, *S. natatoria* biofilms are thin and transparent. If we disrupt the ability of *S. natatoria* to coaggregate, adhesion and growth of binary biofilms is lost (Min, Ricard, 2009). A potentially favorable combination for the formation of binary biofilms is the combination of *M. luteus* and *C. albicans* isolated from the surface of vocal prostheses (Kania et al., 2010): *M. luteus* cells adhere strongly to the surface of *C. albicans* cells. Likewise, coaggregation of *M. luteus* and *Brevundimonas lenta* cells isolated from fouling of the shower heads (Vornhagen et al., 2013) proceeds well. There is evidence that *M. luteus* isolated from venous ulcers on the legs dies in the presence of *P. aeruginosa* or *S. aureus*, isolated therefrom, but survives in the presence of *Streptococcus oralis* (Malic et al., 2011). *S. oralis*, in turn, also perishes in the presence of *P. aeruginosa*, but in a binary biofilm with *S. aureus*, both microorganisms grow better than in mono-species (Malic et al., 2011). This again shows the competitive advantages of *P. aeruginosa* over many microorganisms, which explains the dominance of *P. aeruginosa* in a number of cases of infections.

## CHAPTER 2. Biofilm matrix

Extracellular matrix is an integral feature of biofilms. It functions as a shield that protects bacteria from predators and participates to the formation of oxygen and signaling molecules gradients (McDouglaad et al., 2012). Also, the matrix protects against antibiotics and the immune system (Huang et al., 2011).

### 2.1. Organization and components of biofilm matrix

Generally speaking, the extracellular matrix consists of polysaccharides, proteins and extracellular nucleic acids (Dogsä et al., 2013). To date, the following functional components are distinguished in the extracellular matrix: structural, sorption, surface-active, catalytic-active, information, redox-active and nutrient (Marvasi et al., 2010).

#### 2.1.1. Structural component

The structural component of the matrix consists of neutral polysaccharides and forms the carcass of the extracellular matrix. Such polysaccharides are synthesized by many bacteria, for example *P. aeruginosa*, *E. coli*, *Salmonella typhimurium*, *K. pneumoniae* and *Enterobacter aerogenes* (Morikawa et al., 2006; Ryder et al., 2007). Matrix composition is best studied using the example of *Bacillus subtilis* (Vijayabaskar et al., 2011, Chai et al., 2012), which is one of the favorite models in biofilm studies. *B. subtilis* synthesizes neutral polysaccharides during periodic cultivation on a rich nutrient medium (Shih et al., 2010). The most studied neutral polysaccharides of *B. subtilis* are type I and II levans, which are  $\beta$ -2,6-D-polyfructosans (Marvasi et al., 2010) with the presence of  $\beta$ -2-1 bonds in a random order. In addition to the carcass role, levans participate to

the formation of capsules in pseudomonads, which protects them from bacteriophages, and also promote the formation of soil aggregates in the rhizosphere (Marvasi et al., 2010). The levan polymerization in *B. subtilis* corresponds to saccharase (intracellular) enzymes and levansucrase encoded by the *sacB* gene (Shimotsu, Henner, 1986, Dogsa et al., 2013). Both enzymes are  $\beta$ -fructofuranosidases. The *sacB* gene belongs to the *sacB-yveB-yveA* operon (Pereira et al., 2001). The presence of sucrose in the medium stimulates the synthesis of levansucrase (Shimotsu., Henner, 1986). Sucrose stimulates the anti-terminator of SacY, which permits the expression of *sacB* (Dogsa et al., 2013).

The *yhxB* gene encodes a protein similar to the phosphoglucomutase (Lazarevic et al., 2005), responsible for the synthesis of an unknown oligosaccharide on the surface of *B. subtilis* (Marvasi et al., 2010). If in general we talk about extracellular polysaccharides of *B. subtilis*, their synthesis genes are located in the *epsA-O* locus (Dogsa et al., 2013), and their monomers are formed of fructose, galactose, glucose and N-acetylglucosamine (Chai et al., 2012).

A mention should be made of amyloid pili, consisting of the TasA protein, which form intercellular bridges between *B. subtilis* cells (Dogsa et al., 2013). Fimbria TasA is the second major component of the *B. subtilis* matrix (Morikawa et al., 2006). Fimbria from TasA protein is attached to the cell wall with the TapA protein (Romero et al., 2010). Both proteins are encoded by the operon *tapA-sipW-tasA* (Vlamakis et al., 2013). Also, in *B. subtilis*, a BslA protein (formerly called YuaB), consisting of an Ig-like part and an unusual superhydrophobic cap, is found. It forms a thin elastic layer on the surface of biofilms that determines their hydrophobicity and non-wetting properties (Hobley et al., 2013).

### 2.1.2. Sorption component

The sorption component of the matrix is represented by polymers whose task is the sorption of charged molecules (Marvasi et al., 2010). A well-studied representative is the non-toxic biodegradable anionic polymer poly- $\gamma$ -glutamate (PGA), consisting of D- (50-80%) and L- (20-50%) glutamate (Inbaraj et al., 2008). PGA binds  $Mg^{2+}$  and  $Ca^{2+}$  cations, forms salt bridges between the surface and the cell surface and is an important but not a vital factor for the formation of biofilm factors (Marvasi et al., 2010). The synthesis of PGA is not present in all *B. subtilis* strains (Morikawa et al., 2006). In addition to *B. subtilis*, PGA is synthesized in *Bacillus amyloliquefaciens* (Liu et al., 2010), a bacterium that forms biofilms on the surface of fruits. An interesting fact is the synthesis of extracellular mucus by *B. amyloliquefaciens* (Liu et al., 2010), which covers colonies and consists of PGA. The synthesis of PGA in *B. amyloliquefaciens* is associated with the expression of the *ywsC* (Liu et al., 2010) and *ywtB* genes (Liu et al., 2011). The regulatory factor *pbrA*, whose product (the DNA-binding protein PbrA) is the activator of the operons *epsA-O* (synthesis of matrix polysaccharides) and *yqxM-sipW-tasA* (synthesis of the TasA

protein, the bases of amyloid fimbria participating in cell-cell contacts within the biofilm, Liu et al., 2011, Dogsa et al., 2013), is necessary for the formation of biofilms but does not affect the synthesis of PGA (Liu et al., 2011). Moreover, the suppression of *pbrA* expression stimulates the synthesis of PGA (because of the redistribution of citric acid and glucose - the precursors of the synthesis of TasA and PGA - towards PGA (Liu et al., 2011)).

### **2.1.3. Surface-active component**

The surface-active component of the matrix consists in biosurfactants (Marvasi et al., 2010). Biosurfactants are amphiphilic surfactant secondary metabolites: from low molecular weight glycolipids, sophorolipids, rhamnolipids, lipopeptides to high molecular weight proteins, lipopolysaccharides and lipoproteins (Janek et al., 2012). In *B. subtilis*, the most studied matrix surfactants are lipopeptides (Marvasi et al., 2010). In structure, they are divided into three groups: surfactin-like, iturin-like and plipastatin (fengycin)-like (Xu et al., 2013). The synthesis of lipopeptides is non-ribosomal, using a large complex of enzymes (Lin et al., 1999). Lipopeptides play the role of triggering molecules in signal cascades that regulate the formation of biofilms. In *B. subtilis*, the factor of the quorum-sensing system ComX (*comX* gene) activates the synthesis of surfactin, which leads to the activation of matrix synthesis (the mechanism is still unknown, Lopez et al., 2009). Also, surfactin plays an important role in swarming and aggregating of cells into groups on a solid medium (Debois et al., 2008).

In *B. amyloliquefaciens* SQR9, the lipopeptide bacillomycin D stimulates the transcription of the *kinC* gene, which encodes the KinC membrane protein kinase, which regulates the expression of biofilm formation genes (Xu et al., 2013). In addition to the regulatory function, bacillomycin D suppresses the growth of the phytopathogen *Fusarium oxysporum* in the rhizosphere of cucumber plants (Cao et al., 2011). Iturinic and fungycin lipopeptides, in contrast to surfactin, show pronounced antifungal properties (Xu et al., 2013). However, their functions in the formation of biofilms in bacilli have yet to be clarified.

### **2.1.4. Catalytic component**

The catalytically active matrix component is represented by enzymes that modify the matrix polysaccharides, matrix proteins and other components (Marvasi et al., 2010). The transport systems of polypeptides in Gram-negative bacteria are represented by the T1SS-T9SS, Sec and Tra systems (see "Cell factors"). In Gram-positive bacteria, such functions are ensured by a transport system for adhesion polypeptides (Chagnot et al., 2013), Sec (secretion), FEA (flagella export apparatus, Tra (transfer), FPE (imbrilin-protein exporter) and enzyme transport systems (Marvasi et al., 2010), Tat (twin-arginine translocation pathway), ABC and Sec-SRP (signal recognition particle).

Polysaccharide-modifying enzymes are the already mentioned *B. subtilis* levan sucrase, as

well as pectate lyases, xylanases and amylases (Tjalsma et al., 2004). Extracellular proteases can break down extracellular protein in the case of a lack of nitrogen (Marvasi et al., 2010), and *B. subtilis* strains that are defective in extracellular proteases cannot form biofilms (Tjalsma et al., 2004). Extracellular nuclease is an important enzyme, since extracellular nucleic acids are found in many bacteria. In *Vibrio cholerae*, two extracellular nucleases have been identified: Dns and Xds (Seper et al., 2011). They regulate the amount of extracellular DNA and mutations in their genes lead to an increase in the volume of *V. cholerae* biofilms. Dns predominantly works in the early stages of biofilm development, whereas Xds acts at the maturation stage (accumulation of autoinducers and blocking of dns expression, Seper et al., 2011). Also, Dns and Xds are involved in the formation of tree-like structures in *V. cholerae* biofilms. Regulation of the *dns* gene is carried out by factors HapR (repressor) and *vps* (co-regulator, Blokesch & Schoolnik, 2008). Expression of VPS causes Dns to cut extracellular DNA into smaller fragments, which accelerates its degradation (Seper et al., 2011).

#### **2.1.5. Redox-active component**

The redox-active component of the matrix is represented by electron-transport molecules. In *Geobacter*, the redox-active component consists of extracellular cytochromes of c-type and polymers having local redox-active zones (Babauta et al., 2012, Snider et al., 2012), which form electron transfer chains. In *P. aeruginosa*, the redox active pigments of phenazine nature are able to act as antibiotics and virulence factors for eukaryotic cells (Lopez et al., 2010). Phenazines are able to neutralize a wide spectrum of oxidants with a high redox potential (such as Fe (III)) by dropping electrons on them (Wang et al., 2011). Pyocyanin, a pigment of *P. aeruginosa*, also has a redox activity, which disrupts the calcium homeostasis of eukaryotic cells during pathogenesis (Sarkisova et al., 2005), thereby strengthening the carcass bonds in the polysaccharide matrix through calcium bridges.

#### **2.1.6. Nutrient component**

The nutrient component of the matrix can be any substance belonging to its other components. Separately it is necessary to speak about membrane vesicles (Flemming et al., 2007). Their sizes similar to viruses and they are found in biofilms of gram-negative bacteria, being derived from the outer membrane (Schooling, Beveridge, 2006). They contain proteins, lipids, DNA and other compounds (Tashiro et al., 2013) and could perform nutritional functions.

#### **2.1.7. Extracellular DNA**

It is worth mentioning separately the extracellular DNA which is present in the matrix of biofilms. Extracellular DNA (eDNA) is formed as a result of cell lysis in biofilm (Archer et al., 2011), and in itself is an important component of the matrix (de Kievit, 2009). It should be also released by vesicles, although we ignore in which amount. Thus, several functions of extracellular

DNA are distinguished: participation in cell adhesion; source of genetic material for horizontal gene transfer; source of nutrients for living cells; ionic buffering; modulator of cell exit from the biofilm; regulator to biofilm formation (Okshevsky, Meyer, 2013).

At the moment, little is known about the active secretion of DNA. There is evidence that *Neisseria gonorrhoeae* has an active secretion of extracellular DNA in biofilms which appears regulated by the same way than the regulatory system of Type IV pili (Salgado-Pabon et al., 2010). It is now believed that the main source of extracellular DNA in biofilms is cell lysis, which can also be triggered by phages (Okshevsky, Meyer, 2013). In streptococci, the trigger for cell lysis is probably hydrogen peroxide (Zheng et al., 2011). The most often described mechanism for the appearance of extracellular DNA is bacterial autolysis through a mechanism similar to programmed death in eukaryotic cells (Okshevsky, Meyer, 2013). Cell autolysis is carried out by autolysins - enzymes that break down the cell wall and are under the control of quorum-sensing (Okshevsky, Meyer, 2013). In *S. epidermidis*, most of the extracellular DNA is released by the action of the autolysin AtlE (atlE gene, controlled by the *agr* QS-system, Dai et al., 2012). In addition, the *cidA* gene (murein hydrolase) also participates in cell autolysis (Mann et al., 2009). In *S. aureus*, extracellular DNA is released under the effect of autolysin A (AtlA). Beta-lactam antibiotics in subinhibitory concentrations increase its expression, and as a result, DNA-mediated cell aggregation (Kaplan et al., 2012). In *Enterococcus faecalis* autolysis occurs in a minor subpopulation of cells and gelatinase is involved in this process (Thomas et al., 2009). In this "sacrificial" population there is no synthesis of protein counteracting gelatinase and, thus, there is no protection against autolysis. Also, autolysin AtlA, is involved in *E. faecalis* autolysis (Thomas et al., 2009). Cell autolysis in *E. faecalis* is associated with the expression of the quorum-sensing factor  $\sigma 54$  (Vijayalakshmi, Hancock, 2012).

The hypothesis that extracellular DNA participates in cell adhesion is based on the phenomenon of adsorption of eDNA on the cell surface in the form of loops protruding over a cell surface up to 300 nm (Das et al., 2011). This is a sufficient distance to overcome weak repulsive interactions. There is also a hypothesis about the interaction of such extracellular DNA loops with the surface nanorelief (Mitik-Dineva et al., 2008). In the case of distances of the order of several nanometers for extracellular DNA, interaction of electron-donor and electron-acceptor groups of extracellular DNA and the surface was established (Das et al., 2011). Extracellular DNA affects the hydrophobicity of cells, which affects their ability to adhere to the surface by different ways (all depends on the hydrophobicity of the surface, Okshevsky, Meyer, 2013). Some studies show an improvement of cell adhesion to hydrophobic surfaces due to the fact that extracellular DNA increases the hydrophobicity of cells (Das et al., 2011). The positive effect of extracellular DNA on cell adhesion to abiotic substrates was demonstrated in *S. epidermidis* (glass, polystyrene, Qin et al.,

2007) and *Acidovorax temperans* (glass, Heijstra et al., 2009). In *S. epidermidis*, extracellular DNA and extracellular matrix binding protein (Embp) are key agents of an alternative way of biofilm formation independent of polysaccharide extracellular adhesin (Doroshenko et al., 2014). In *L. monocytogenes*, extracellular DNA is important at the initial stage of adhesion. Treatment of extracellular DNA-deprived culture with cellular DNA does not restore the ability to adhere (Harmsen et al., 2010).

The importance of extracellular DNA as a structural component of the biofilm matrix was demonstrated in experiments on DNAase treatment of *E. coli* biofilms and their subsequent DNA processing (growth suppression and stimulation respectively, Liu et al., 2008). In *Caulobacter crescentus*, motile cells emerging from the biofilm for dispersal have a stalk at one pole for attachment to the substrate. Extracellular DNA associated with the stalk effectively prevents cell adhesion to pre-existing biofilms (Berne et al., 2010). The amount of DNA in the matrix depends on the species and even bacterial strain (Okshevsky, Meyer, 2013). It is considered that extracellular DNA is present in the early stages of biofilm growth (Seper et al., 2011). However, *Streptococcus pneumoniae* shows an opposite behavior with eDNA being isolated in the stationary growth phase (Moscoso & Claverys, 2004). Therefore, with the importance of extracellular DNA for the formation of biofilms, there is no correlation between the growth of biofilms and the amount of extracellular DNA in them. It is assumed that extracellular DNA binds to matrix molecules through nonspecific electrical interactions and, perhaps, divalent cations contribute to this (Okshevsky, Meyer, 2013). In *Myxococcus xantos*, mutants that do not form exopolysaccharides do not retain extracellular DNA (Hu et al., 2012). *N. meningitides* has 2 DNA-binding proteins expressed on its surface: the Nhba antigen and the IgA protease a-peptide (Arenas et al., 2013). Removal of these proteins by the protease NaIP (autotransporter) leads to a decrease in the growth of biofilms. Interaction of extracellular DNA with double-layer membrane vesicles in biofilms of Gram-negative bacteria was also shown (Schooling et al., 2009). The relationship of the *E. coli* toxin-antitoxin system to extracellular DNA was demonstrated: the *hipA* gene mutants form less biofilms, with less extracellular DNA and less sensitivity to DNase (Zhao et al., 2013). The mechanism of the phenomenon is not yet clear. Extracellular DNA binds to type IV pili in *P. aeruginosa*, which is associated with the formation of a cap (the location of release of mobile cells) on the top of mushroom-like biofilms (Barken et al., 2008). Non-protein molecules can also bind to extracellular DNA. In *P. aeruginosa*, pyocyanin (an electron acceptor with NADH during oxygen deficiency) binds extracellular DNA, which probably stimulates intercellular interactions (Das et al., 2013).

Extracellular DNA participates to horizontal genes transfer in biofilms. To date, it has been established that horizontal gene transfer occurs more efficiently within biofilms (Roberts et al.,



2006). As a factor in the output of extracellular DNA into the matrix, the competence of cells was examined. This was demonstrated in *S. pneumoniae*, in which the murein hydase CbpD (choline binding protein D) is a key competence inducer, and *cbpD* mutants do not practically secrete extracellular DNA (Okshevsky, Meyer, 2013). *S. mutans* demonstrated the importance of the *comA*, *comB*, *comC*, *comD* and *comE* competence genes in the formation of biofilms (Li et al., 2002). In multi-species biofilms, the release of eDNA and competence is regulated by the antagonism of the species (Okshevsky, Meyer, 2013). With all of this, horizontal genes transfer is not the only cause of extracellular DNA release. This is proven in *S. pneumoniae*, in which extracellular DNA is present in the matrix for a long time after the disappearance of cell competence (Okshevsky, Meyer, 2013).

In addition, extracellular DNA performs a protective function by binding antimicrobial compounds and thus preventing their penetration into the cells. Thus, extracellular DNA binds vancomycin, which protects biofilms of *S. epidermidis* from the action of subinhibitory concentrations of the antibiotic (Doroshenko et al., 2014). The affinity of extracellular DNA to vancomycin is a hundred times higher than that of its intracellular target, the peptide D-Ala-D-Ala (Doroshenko et al., 2014). In general, DNA, forms negatively charged polyions and acts like a molecular trap for positively charged antimicrobial molecules, such as, for example, antibiotics of aminoglycoside and peptidic nature (Das et al., 2013).

Thus, extracellular DNA performs informative, nutritional, structural, regulatory, protective and adhesive roles, therefore, it can be included in the composition of different matrix components.

## **2.2. The matrix of biofilms of some skin microorganisms**

In this section the composition of the matrix of microorganisms used in thi thesis will be described.

### **2.2.1. *Pseudomonas***

The biofilm matrix of *Pseudomonas* and particularly that of *P. aeruginosa* was studied in detail. Briefly: its main components are polysaccharides, polysaccharide-containing compounds, extracellular DNA and proteins (Mann & Wozniak, 2012).

Basic polysaccharides according to Mann & Wozniak, 2012 (if available, evidence for the presence of polymers in other *Pseudomonas* species is given):

1) alginate (L-guluronic acid, D-mannuronic acid, connected by  $\beta$ -1,4-bond) is one of the main polymers of *P. aeruginosa* matrix. It retains water and nutrients in the biofilm. Also found in *P. fluorescens* (Maleki et al., 2015), *P. chlororaphis* (Chen et al., 2015a) and *P. putida* (Chang et al., 2007) biofilms;

2) levan (branched  $\beta$ -polyfructan), serving as a supply of nutrients in case of starvation. Also found in *P. chlororaphis* (Visnapuu et al., 2011) and *P. fluorescens* (Mann, Wozniak., 2012)

biofilms;

3) Psl (polymer D-mannose, L-rhamnose and D-glucose) - a polymer in two forms: soluble, relatively low molecular weight, and high molecular weight, associated with cells. Function - adhesion of cells to each other and to the surface, and, possibly, signal transmission. It is present in *P. fluorescens*.

4) Pel (a polymer rich in N-acetylglucosamine and N-acetylgalactosamine, Marmont et al., 2017), involved in the process of coaggregating cells with each other and with matrix components, capturing antibiotic molecules (functions similar to that of extracellular DNA). The operon *pel* is partially present in *P. fluorescens* and *P. putida*;

5) Cellulose - plays a role in the formation of biofilms at the interface of the "fluid-air" phases. It is present in *P. fluorescens*.

The protein component of the matrix of pseudomonads is a variety of adhesins (Large adhesion protein (Lap), responsible for irreversible cell adhesion to the surface, Mann & Wozniak, 2012), pili (especially type IV), extracellular lectins (LecA, LecB, having a cytotoxic effect on eukaryotic cells) and extracellular enzymes (Mann & Wozniak, 2012). Rhamnolipids - compounds consisting of monosaccharides and fatty acid molecules and having surfactant functions, antibacterial and fungicidal activity, form an important part of the secretion of pseudomonads (including in *P. chlororaphis* (Gunther et al., 2005)). Probably, because rhamnolipids, *Pseudomonas* can use high-molecular hydrophobic compounds as a source of energy (Mann, Wozniak, 2012). For extracellular DNA and its functions, see section 1.5.1.7.

In addition to basic compounds, *Pseudomonas* matrix contains gases, water, metabolites and signal molecules (Mann, Wozniak, 2012). Since the matrix of *Pseudomonas* biofilms has been studied in depth, the reader can refer to sources describing its composition and functions in more detail: for example, to the monograph of Ramos et al., 2015.

### **2.2.2. *Staphylococcus***

The main components of the biofilm matrix of *S. aureus* and *S. epidermidis* are polysaccharides, proteins, extracellular DNA (Schilcher et al., 2016). The main polysaccharide component of the biofilm matrix in both staphylococci is poly- $\beta$ -(1,6)-N-acetyl-D-glucosamine (PNAG), which is sometimes referred to as surface polysaccharide adhesin. It is the main element of the matrix responsible for cell adhesion and coadhesion, protection from the immune system and organization of a three-dimensional biofilm structure in *S. aureus* and *S. epidermidis* (Izano et al., 2008; Gökçen et al., 2013). The operon coding for the synthesis of PNAG in staphylococci is *icaADBC* (Gökçen et al., 2013). The *icaB* gene is responsible for the deacetylation of PNAH (about 16% of the monomers), leaving the remaining glucosamine residues bearing a positive charge and making the PNAGH more cationic. This is important for electrostatic interactions with surfaces, and

also to protect against the action of the immune system by repelling positively charged antimicrobial peptides (Gökçen et al., 2013). Analogues of PNAG are synthesized in a number of other bacteria, including *P. fluorescens*, in which it performs the function of adhering cells to surfaces and to each other, protecting them from detergents and antibiotics (Izano et al., 2008).

The second important component of the matrix of biofilms of staphylococci is extracellular DNA. It is interesting that PNAG and extracellular DNA differently participate in the formation of the biofilm matrix of *S. epidermidis* and *S. aureus*: in *S. aureus*, extracellular DNA is the main component of the matrix, and PNAG is minor, whereas in *S. epidermidis* it is the opposite (Izano et al., 2008). This was shown by treatment of biofilms with dispersin B and DNase I: dispersin B (an enzyme that cleaves PNAG) destroyed biofilms of *S. epidermidis*, but did not destroy biofilms of *S. aureus*; DNase I also destroyed *S. aureus* biofilms, but did not destroy biofilms of *S. epidermidis* (Izano et al., 2008). By the same way, in *S. epidermidis* there is an inverse correlation between PNAG and eDNA: in strains actively synthesizing PNAG, there is little extracellular DNA in the matrix, and in many strains with low PNAG production, extracellular DNA is abundant (Campoccia et al., 2011). Thus, extracellular DNA is a key component of the matrix involved in the alternative, PNAG-independent formation of *Staphylococcus* biofilms, since PNAG-deficient strains do not lose the ability to form biofilms (Izano et al., 2008; Campoccia et al., 2011; Schilcher et al., 2016).

Teichoic and lipoteichoic acids on the cell surface are another component of the biofilm matrix of *S. epidermidis* (Otto, 2012) and *S. aureus* (Weidenmaier et al., 2001; Ahn et al., 2018). Teichoic acids are linked by covalent bonds to the peptidoglycan of the cell wall, whereas lipoteichoic acids do not have covalent binding to the cell wall molecules (Otto, 2012). Teichoic acids of *S. epidermidis* consist in a polyglycerol phosphate chain substituted (bond to the second atom of glycerol) by  $\alpha$ -glucose,  $\alpha$ -glucosamine, D-alanine,  $\alpha$ -6-D-alanylglucose (Otto, 2012). Teichoic acids of *S. aureus* consist in a ribitolphosphate or glycerol phosphate chain (lipoteichoic acids) substituted with N-acetylglucosamine and D-alanine residues (Gross et al., 2001). Lipoteichoic acids are fixed on the cell surface with a glycolipid anchor: dihexosyl-diacylglycerol or dihexosyl-triazylglycerol (Ahn et al., 2018). Teichoic acids are involved in the processes of cell adhesion to the surface and to each other, mutant strains of staphylococci that do not have enough teichoic acids have increased hydrophobicity, resulting in a weakening of biofilm formation (Fedtke et al., 2007). Teichoic acids are involved in the adhesion of *staphylococcus* cells to fibronectin, and in inflammatory processes (Otto, 2012) through interaction with the TLR2 receptor (Ahn et al., 2018).

The matrix of staphylococci also contains proteins. Biofilm attachment protein (Bap) is a *S. aureus* protein associated with a cell wall and consisting of several repeating domains (Taglialegna et al., 2016). Bap is responsible for the adhesion of cells to the surface and to each other at the

initial stages of biofilm formation and, together with extracellular DNA, it is a component of the PNAG-independent way of biofilm formation (Taglialegna et al., 2016). Bap is also found in *S. epidermidis*, which in addition to Bap has its homologue Bhp (Bap homologue protein), which contributes to the formation of biofilms, however, it is considered less important for the formation of biofilms (Otto, 2012; Taglialegna et al., 2016). Embp (extracellular matrix binding protein) is a large (460 kDa) surface protein involved in the initial adhesion of cells, as well as cell adhesion to fibronectin in *S. epidermidis* (Otto, 2012). Embp molecules form rod-shaped structures on the surface of cells (Buttner et al., 2015). *S. aureus* has an Embp-Ebh homolog with similar functions and parameters, including also adhesion to fibronectin (Buttner et al., 2015). If Embp in *S. epidermidis* is primarily responsible for the adhesion and coagulation of the cells at the initial stage of biofilm development, the accumulation-associated protein (Aap) plays a role in addition to the initial adhesion and co-adhesion function at later stages of biofilm development (Otto, 2012; Buttner et al., 2015). Aap in biofilm undergoes processing with proteases to obtain an active form, exists predominantly in position with the C-terminus attached to the cell wall and forms pili of 120 nm, which permeate biofilm and bind cells to each other, as well as to the surface (Otto, 2012; Buttner et al., 2015). Aap is considered as a key molecule in the process of PNAG-independent biofilm formation in *S. epidermidis* (Buttner et al., 2015). In addition, the biofilm formation of *S. epidermidis* involves the surface protein SesC (*Staphylococcus epidermidis* surface protein), and in *S. aureus* the surface proteins SasC and SasG (*Staphylococcus aureus* surface protein), adhesion factor B (ClfB) SdrC (serine aspartate repeat protein) and fibronectin binding proteins of FnBPA and FnBPB (Speziale et al., 2014). The latter refer to the so-called MSCRAMM (microbial surface component recognizing adhesive matrix molecules), the virulence factors responsible for attachment to connective tissue proteins (fibronectin, collagen, Kang et al., 2013; Speziale et al., 2014).

### **2.2.3. *Cutibacterium acnes***

Two published articles were devoted to the study of the matrix of *C. acnes* biofilms at the time of this work writing: Jahns et al., 2016 and Okuda et al., 2018. They will be considered in detail in the discussion section. Here it is worth indicating that to date it is known that the matrix of *C. acnes* consists of polysaccharides, proteins and carbohydrates, and one of the carbohydrates of the *C. acnes* matrix is PNAG (Okuda et al., 2018).

### **2.2.4. *Micrococcus luteus* and *Kytococcus schroeteri***

At the time of this thesis writing, only one article has been found devoted to the study of the extracellular matrix of *M. luteus*. This article (Mauclair, Egli, 2009), showed that the matrix of *M. luteus* biofilms consists predominantly of polysaccharides and proteins. However, in the literature in-depth studies of the matrix of *M. luteus* have not been found to date. Articles devoted to the study

of the matrix of *K. shroeteri* at the time of writing this work were not found. On the basis of known data on other microorganisms, it can be assumed that the *K. schroeteri* matrix should also consist of proteins, carbohydrates and extracellular DNA. This makes the work in this direction perspective and relevant. It is possible that, being actinomycetes, both microorganisms synthesize PNAG like *C. acnes*. There are some data on the protein Rpf, secreted by *M. luteus*, which affects the intercellular contacts of *M. luteus* and *Mycobacterium smegmatis* (Nikitushkin et al., 2011). Rpf (resuscitation-promoting factor) is secreted by *M. luteus* in the medium and stimulates the release of cells from an inactive state and their proliferation (Su et al., 2015). This makes the use of the culture liquid of *M. luteus* a promising tool, for example, to stimulate the growth of bacteria involved in biphenyl compounds decomposition (Su et al., 2015). However, there is no evidence for a role of Rpf in the growth of *M. luteus* biofilm.

## *Experimental study*

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EXPERIMENTAL  
ARTICLES

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## Regulation of Biofilm Formation by *Pseudomonas chlororaphis* in an in vitro System

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**Abstract**—The mutants of *Pseudomonas chlororaphis* 449 with completely or partially suppressed accumulation of *N*-acyl homoserine lactones exhibited the absence or a pronounced decrease of their capacity for stimulation of biofilm growth in the presence of azithromycin. Biofilms of the wild type strain preformed in the presence of the stimulatory azithromycin concentrations exhibited more intense staining with a polysaccharide-specific dye 1,9-dimethyl methylene blue (DMMB) and were more resistant to heat shock. These findings indicate accumulation of the structural matrix polysaccharides, which play a protective role under conditions of thermal shock. Extremely low azithromycin concentrations (0.001–0.01 µg/mL) inhibit biofilm formation by *P. chlororaphis* 449 and *P. chlororaphis* 66 with suppression of the synthesis of DMMB-staining polysaccharides.

**Keywords:** biofilms, antibiotics, activation of biofilm formation, quorum sensing

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A number of regulatory systems participating in formation of bacterial biofilms rely on such components as cyclic diguanosine monophosphate (c-di-GMP), “small” RNA (sRNA), indole derivatives, and other substances [1]. The system of “quorum sensing” (QS) is one of the most important systems of global metabolic regulation, involved in biofilm formation.

The functioning of QS system in gram-negative bacteria is mediated by two types of proteins. One type is responsible for the synthesis of signal molecules commonly referred as autoinducers, usually *N*-acyl homoserine lactones (AHL). The second type of the proteins interacts with autoinducers, launching transcription of specific genes [2–4]. Over 70 genera of the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -proteobacteria are known to use AHL-involving QS [5]. In *Vibrio fischeri*, AHL synthesis is controlled by the *luxI* gene encoding AHL-synthase LuxI. When a critical concentration threshold is reached, AHL binds to the LuxR transcription activator and transcription of QS-dependent genes, including *luxI*, is launched. Thus, the concentration of signal molecules in the medium has an avalanche-like increase. Since AHL are able to diffuse inside and outside of cells, the AHL pool correlates with cell density, providing control of gene expression depending on the population density. At least three QS systems operate in pseudomonads. The most studied systems, LasIR and RhlIR (LuxIR system homologues), use AHL as signal molecules, while the third system, AQ, is alkyl quinolone-dependent [6, 7].

Although involvement of AHL-dependent QS-system in biofilm formation was demonstrated in several studies, molecular mechanisms of these processes remain incompletely understood. It was shown recently that inactivation of the AHL synthase gene *cvrI* in *Chromobacterium violaceum* leads to impaired synthesis of the components of the polysaccharide extracellular polymer matrix (EPM), accompanied by increased sensitivity of the biofilms to extreme environmental conditions and antibiotics [8, 9].

The goal of the present work was to study the effect of low (sub-bacteriostatic) concentrations of azithromycin on *P. chlororaphis* wild type strains, modified strains, and mutants with defects in some global regulatory systems, in order to reveal the role of those systems in the regulation of biofilm formation by azithromycin.

### MATERIALS AND METHODS

**Subjects of study.** The subjects of study were pure cultures of gram-negative saprotrophic bacteria *P. chlororaphis* (strains 66 and 449), modified strains, and mutants of the latter strain, obtained in the laboratory of Expression regulation of genes in microorganisms, Institute of Molecular Genetics, Russian Academy of Sciences (IMG RAS) and stored in collection of IMG RAS. The basic characteristics of those microorganisms are summarized in the table.

**Cultivation and storage of microorganisms.** The microorganisms were stored on semiliquid (0.3%

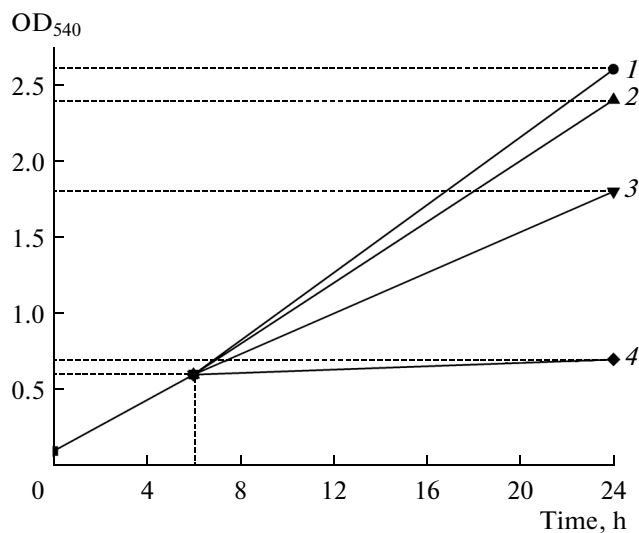
<sup>1</sup> Corresponding author; e-mail: plakunov@inmi.host.ru

## Features of bacteria used in the study

Bacterial strains	Characteristics of the strains	Source
<i>P. chlororaphis</i> 449	Prototroph; able to synthesize AHL	Collection of the Institute of Molecular Genetics, Russ. Acad. Sci.
<i>P. chlororaphis</i> 66	Prototroph; able to synthesize AHL	Collection of the Institute of Molecular Genetics, Russ. Acad. Sci.
<i>P. chlororaphis</i> 449, mutant 2	Mutation in the <i>rpoS</i> gene; able to synthesize AHL	[10]
<i>P. chlororaphis</i> 449, mutant 3	Mutation in the <i>gacS</i> gene; ability to synthesize AHL is decreased	[11]
<i>P. chlororaphis</i> 449, mutant 4	Mutation in the <i>phzB</i> gene; able to synthesize AHL	[11]
<i>P. chlororaphis</i> 449, mutant 5	Mutation in the <i>phzA</i> gene; able to synthesize AHL	[11]
<i>P. chlororaphis</i> 449/pME6000, strain 6e	Contains a vector plasmid pME6000, Tc-r; able to synthesize AHL	[11]
<i>P. chlororaphis</i> 449/pME6000, strain 7e	Contains plasmid pME6863, including the cloned gene <i>aiiA</i> , Tc-r; is unable to accumulate AHL	[11]

agar) LB medium in agar columns under paraffin oil at 4–6°C. In case the of the mutants 2, 3, 4, and 5, sterile kanamycin solution (final concentration 100 µg/mL) was added to the medium for storage. In the case of strains 6e and 7e, sterile tetracycline solution was used (final concentration up to 40 µg/mL). Bacteria were grown in liquid LB medium at 29–30°C on a shaker (150 rpm) for 20–24 h. These cultures were used as inocula for biofilm experiments.

The biofilms were obtained and their sensitivity to inhibitors was studied as described previously [9].

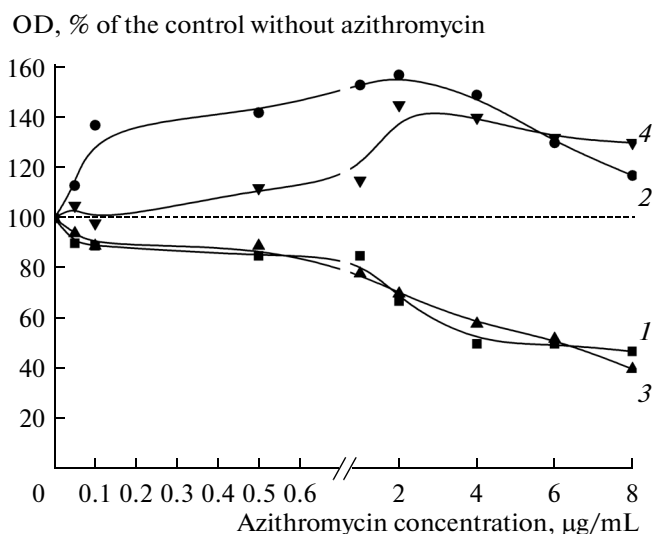


**Fig. 1.** Graph illustrating a method of calculating of *P. chlororaphis* 449 planktonic culture sensitivity to heat shock: 29°C (1), 34°C (2), 37°C (3), and 45°C (4).

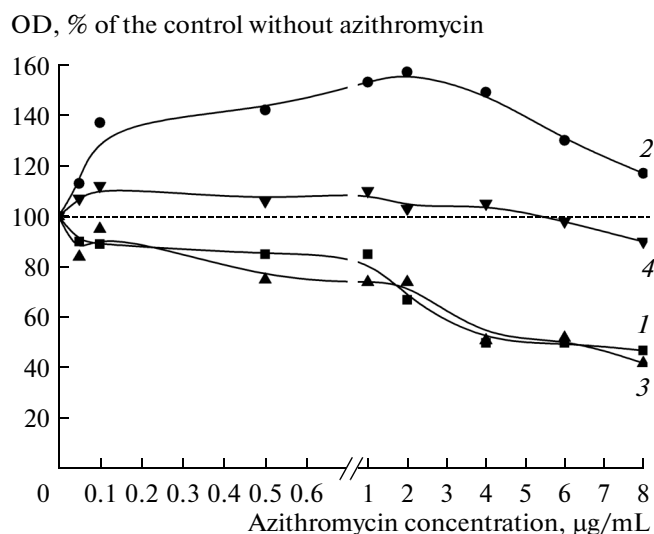
**Effect of heat stress on growth of biofilms of *P. chlororaphis* 449 in the presence of azithromycin.** The sensitivity of planktonic cultures and biofilms to heat stress was characterized as the ratio of increase of a parameter proportional to the biomass of a studied object (optical density or light scattering) in the presence and absence of azithromycin at studied temperatures to its increase at the optimal temperature (29°C). The calculation method is shown on Fig. 1 for *P. chlororaphis* 449 planktonic culture without the antibiotic. The culture was incubated for 6 h, the time required for development of the “biofilm” phenotype and beginning of matrix formation [8]. In this case, the optical density (OD<sub>540</sub>) determined as light scattering by suspensions of 6-h planktonic cells reached the value of 0.6. After transfer of the culture to experimental temperature conditions, the incubation continued for another 24 h. Increase of the optical density of planktonic cell suspension at 29°C was 2.6 – 0.6 = 2.0 unit OD. This value was accepted as 100%. Culture growth at other temperatures was assessed similarly. For example, at 37°C, it was 1.6 – 0.6 = 1.0 units OD. Thus, the relative culture growth at this temperature was  $1.0/2.0 \times 100 = 50\%$ . This value was plotted on the graphs (Figs. 2–9). The relative growth of biofilms was calculated similarly (based on optical density of the dyes extracted from preliminary stained biofilms). The measurements were conducted at 590 and 670 nm for crystal violet (CV) and 1,9-dimethyl methylene blue (DMMB), respectively [9].

**Microscopy.** For phase contrast microscopy (PCM) studies and epifluorescence microscopy (EFM) Axio Imager, D1 Carl Zeiss, objective lens ×40 microscope was used. Biofilms were formed on microscopic slides. For this purpose, standard slides were cut into three parts and were cleaned by incubation in

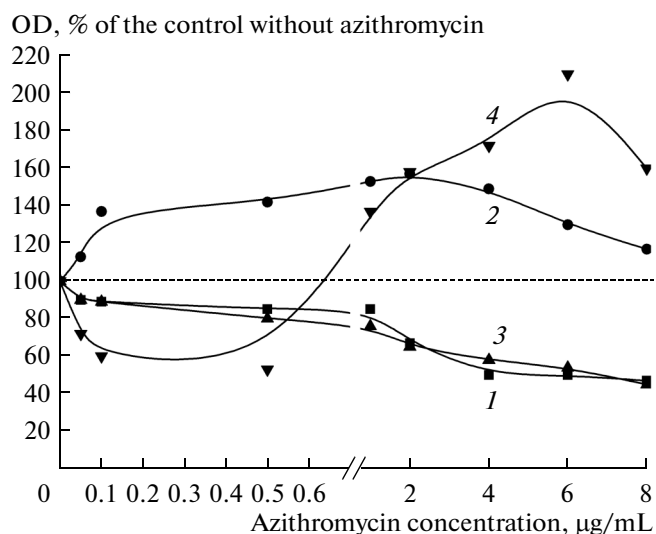




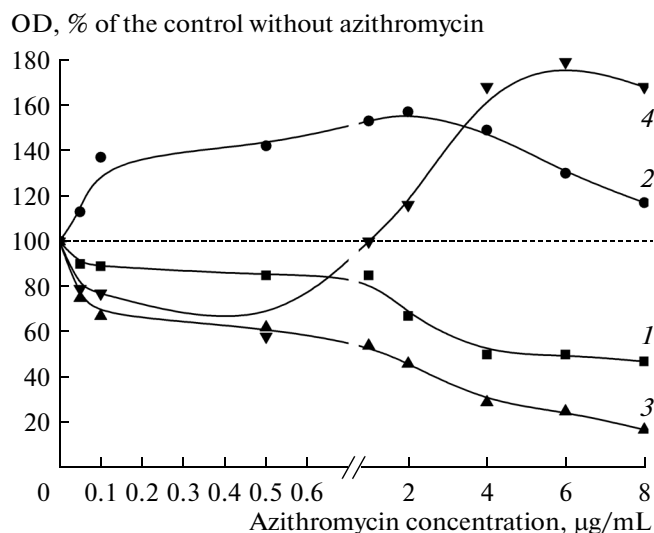
**Fig. 2.** Growth of planktonic cultures (1, 3) and biofilms (2, 4) of *P. chlororaphis* 449 (1, 2) and mutant 2 (3, 4) at different azithromycin concentrations. Biofilms stained with CV. On this and following figures a dashed line indicates the control level without addition of the antibiotic.



**Fig. 3.** Growth of planktonic cultures (1, 3) and biofilms (2, 4) of *P. chlororaphis* 449 (1, 2) and mutant 3 (3, 4) at different azithromycin concentrations. Biofilms stained with CV.



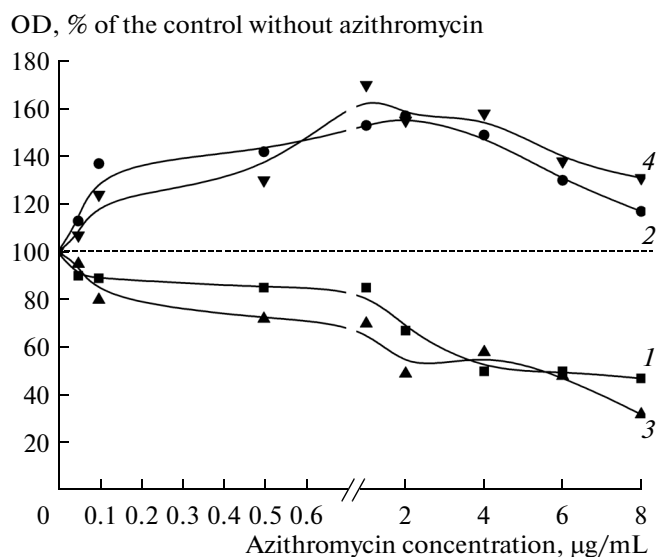
**Fig. 4.** Growth of planktonic cultures (1, 3) and biofilms (2, 4) of *P. chlororaphis* 449 (1, 2) and mutant 4 (3, 4) at different azithromycin concentrations. Biofilms stained with CV.



**Fig. 5.** Growth of planktonic cultures (1, 3) and biofilms (2, 4) of *P. chlororaphis* 449 (1, 2) and mutant 5 (3, 4) at different azithromycin concentrations. Biofilms stained with CV.

chromic mixture for a day. After washing, the slides were sterilized in tubes with 5 mL LB medium (1 atm), which were then inoculated with 50  $\mu$ L of the culture. After 24 h of cultivation, the planktonic cell suspension was decanted, the slides were dried, and the biofilms were fixed by heating gently in a gas burner flame. For PCM, the biofilms were stained with DMMB according to [12], with some modifications. To obtain stock solution of the dye, 20 mg DMMB (double salt with zinc chloride) was dissolved in 5 mL

96% ethanol. The stock solution of 0.2 g sodium formate (anhydrous) and 0.2 mL 99% formic acid in 95 mL distilled water (pH 3.3) were prepared separately. After mixing, the solution, which is stable for several months at room temperature, was stored in the dark. For EFM, DAPI [13] and the fluorescent stain FilmTracer™ SYPRO Ruby biofilm matrix stain (according to the manufacturer's instructions) [14] were used.



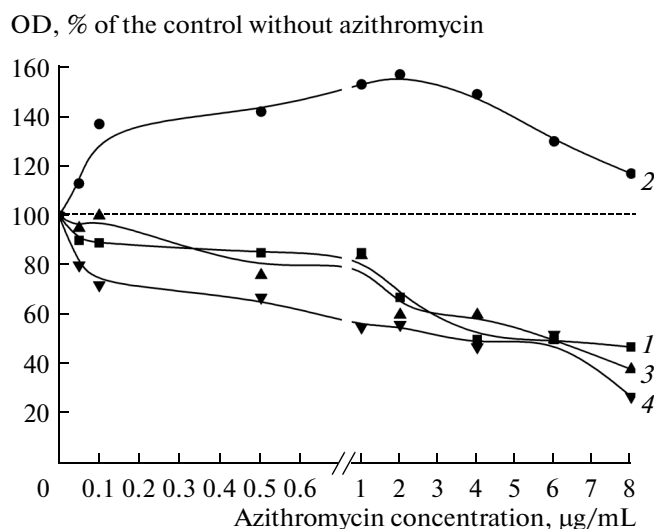
**Fig. 6.** Growth of planktonic cultures (1, 3) and biofilms (2, 4) of *P. chlororaphis* 449 (1, 2) and strain 6e (3, 4) at different azithromycin concentrations. Biofilms stained with CV.

**Statistical reliability** of the results is based on selection of a typical experiment by the nonparametric method of comparing paired data, taking into account “the sign test” [15]. As a result of four or more statistical variants for each sample, the median of relative values (in %) was chosen as an average value. Graphical data were processed in the Origin 8.6 software package using B-spline function.

## RESULTS AND DISCUSSION

To study the regulatory patterns of biofilm formation regulation in *P. chlororaphis* 449, we chose the mutants with an interruption in several systems of global metabolic regulation that take part in biofilm formation regulation [16]: (a) in the gene *rpoS* encoding the sigma S subunit of RNA polymerase, RpoS (mutant 2); (b) in the two-component global regulation system GacA-GacS (mutant 3); (c) in the *phz* operon system, encoding the synthesis of phenazine antibiotics (mutants 4 and 5). We also used the strain *P. chlororaphis* 449, transformed by the plasmid pME6863, which contained the cloned heterologous gene *aiiA* encoding homoserine lactonase. This enzyme breaks down AHL, the signal molecules of the QS systems [17]. As a result, it disturbs the functioning of QS regulation (strain 7e). For comparison, strain 6e was used, containing the vector plasmid pME6000 which does not influence AHL synthesis and bacterial cell migration on solid surfaces (see table).

Our experiments were aimed at investigation of the effect of these mutations on the patterns of biofilm formation by *P. chlororaphis* 449. The approach chosen for the purpose was based on the use of a macrolide antibiotic azithromycin as a biochemical tool.

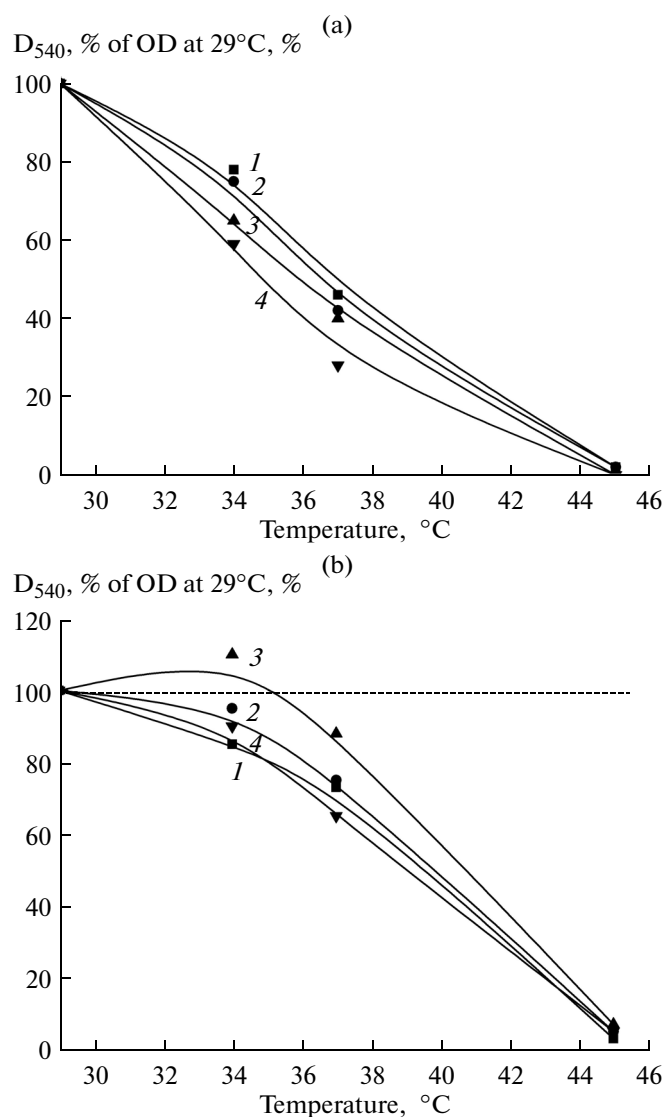


**Fig. 7.** Growth of planktonic cultures (1, 3) and biofilms (2, 4) of *P. chlororaphis* 449 (1, 2) and strain 7e (3, 4) at different azithromycin concentrations. Biofilms stained with CV.

Azithromycin is a macrolide antibiotic widely used for clinical treatment of biofilm-related infections [18].

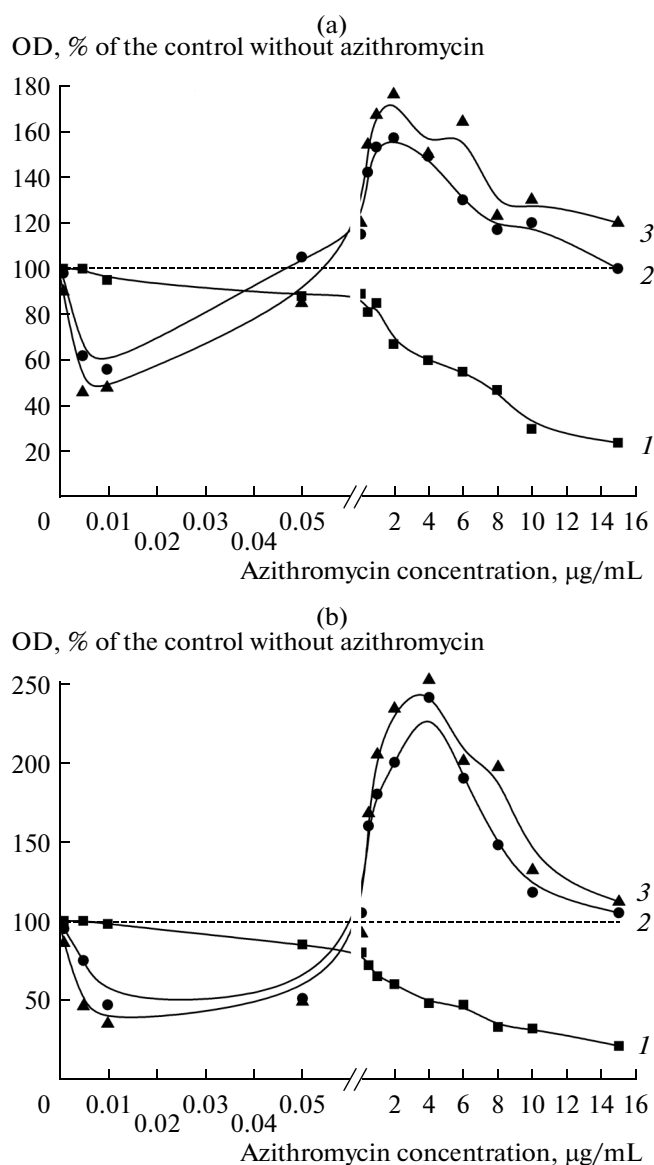
Previously, we used azithromycin to study the dynamics of biofilm formation. It was found that low, sub-bacteriostatic concentrations of this antibiotic could significantly activate the growth of biofilms of several gram-positive bacteria [19]. It was important to determine whether azithromycin had the same effect on gram-negative bacteria *P. chlororaphis* 449 and whether the disorders in the regulatory systems mentioned above affected this effect.

**Azithromycin effect on *P. chlororaphis* 449 wild type and mutant strains.** In mutant 2, the *rpoS* gene, which encodes the sigma S subunit of RNA polymerase, is inactivated, resulting in suppression of the synthesis of phenazine antibiotics with no significant effect on the synthesis of four type of *N*-acyl homoserine lactones (*N*-butanoyl-L-homoserine lactone, *N*-hexanoyl-L-homoserine lactone, *N*-(3-oxo-hexanoyl)-L-homoserine lactone and a minor AHL) or on bacterial ability to move over the surface of a dense medium (swarming) [10]. The effect of azithromycin on planktonic culture and biofilm growth of this mutant (in comparison with the same parameters for the wild type) are presented on Fig. 2. It can be seen that growth of planktonic cultures of both strains is almost equally sensitive to azithromycin. Low concentrations of azithromycin (0.1–1 µg/mL), which do not significantly influence the growth of planktonic cultures, significantly stimulate (1.4 to 1.6 times) the growth of biofilms of both strains, similarly to what was shown previously for gram-positive bacteria [19]. Thus, mutation of the *rpoS* gene does not significantly influence (qualitatively) the nature of the action of azithromycin.



**Fig. 8.** Growth of planktonic culture (a) and biofilm (b) of *P. chlororaphis* 449 at different temperatures. Without antibiotic (1); in the presence of azithromycin: 6 µg/mL (2), 8 µg/mL (3), and 15 µg/mL (4). Biofilms stained with CV.

In mutant 3, the two-component system GacA-GacS, which regulates the synthesis of all types of AHL, phenazine antibiotics, exoproteases, polygalacturonase, pectin methyl esterase, and antagonistic activity over phytopathogenic fungi, is impaired. The mutation is localized in the *gacS* gene encoding sensor kinase GacS. This mutant retains the capacity for synthesis of only some AHL types, and the level of synthesis is lower than in the wild type [11]. As can be seen from Fig. 3, planktonic cultures of these strains do not differ in their sensitivity to azithromycin. This mutant retained the tendency for stimulation of biofilm formation by low concentrations of azithromycin,



**Fig. 9.** Effect of low concentrations of azithromycin on planktonic culture (1) and biofilm (2, 3) growth of *P. chlororaphis* 449 (a) and *P. chlororaphis* 66 (b). Biofilms stained with CV (2) or DMMB (3).

though it is much less pronounced than in the wild type strain. It is obvious that the set (or level) of AHL serves as an essential factor for development of this effect.

The mutants 4 and 5 were obtained by transposon mutagenesis (using mini-Tn5Km) and contain inactivated genes of the phenazine operone: *phzB* (mutant 4) and *phzA* (mutant 5). As a result of the absence of phenazine synthesis, the mutants do not have the orange color typical of the wild type strain. However, synthesis of the complete set of AHL is preserved in this mutant [11].

Figs. 4 and 5 demonstrate the effects of azithromycin on the growth of planktonic cultures and biofilms of mutants 4 (Fig. 4) and 5 (Fig. 5). The sensitivity to azithromycin in planktonic cultures of mutants 4 and 5 was close to that in planktonic cultures of the wild type strains (mutant 5 was slightly more sensitive). While the stimulation of biofilm growth by sub-inhibitory concentrations of the antibiotic was observed for both mutants, it developed at higher concentrations (1–8  $\mu\text{g/mL}$ ). In the lower concentration region (0.05–0.5  $\mu\text{g/mL}$ ), considerable inhibition of this process was observed. The role of this phenomenon will be discussed further.

Strain 7e contains the plasmid pME6863, which includes the cloned gene of *N*-acyl homoserine lactonase (AiiA) responsible for the degradation of all types of AHL produced by this strain; degradation is accompanied by a drastic decrease in phenazine synthesis and impaired ability of bacterial cells to swarm. As we have already mentioned, strain 6e bears a vector plasmide pME6000, which does not affect AHL synthesis [20].

Figures 6 and 7 illustrate the influence of azithromycin on growth of planktonic cultures and biofilms of strains 6e (Fig. 6) and 7e (Fig. 7). It is obvious that introduction of the plasmid pME6000 (strain 6e) which does not lead to AHL synthesis decrease, affected significantly neither the azithromycin sensitivity of the planktonic culture nor the stimulating effect of this antibiotic on biofilms (Fig. 6). On the contrary, strain 7e, which contains the plasmid pME6860 inducing AHL degradation, fully lost its capacity for the stimulation of biofilm formation in the presence of low azithromycin concentrations. Moreover, biofilm growth became slightly more sensitive to effects of this antibiotic than the growth of planktonic culture (Fig. 7). Thus, according to the results of our experiments, the stimulatory effect of low concentrations of azithromycin on biofilm formation positively correlates with capacity for AHL synthesis. This correlation will be discussed below.

**Sensitivity to heat stress.** There is evidence that azithromycin and other macrolide antibiotics in sub-inhibitory concentrations stimulate the synthesis of biofilm matrix polysaccharides in clinical strains of *Staphylococcus epidermidis* [21]. On the other hand, our previous studies confirmed an important role of the polysaccharide matrix of the biofilms of several gram-positive and gram-negative saprotrophic bacteria in resistance not only to biocides, but also to such environmental stress factors as heat and acidic or osmotic shock [8].

If stimulation of *P. chlororaphis* 449 biofilm formation by azithromycin implied enhanced synthesis of the polysaccharide matrix, then such “stimulated” biofilms might be more resistant to heat. To verify this assumption, we studied the sensitivity of *P. chlororaphis* 449 biofilms to heat stress in the presence of stimulatory concentrations of azithromycin.

According to the results shown on Fig. 8, sensitivity of planktonic cultures to heat shock in the presence of azithromycin slightly increased (Fig. 8a). On the contrary, the sensitivity of biofilms to heat stress in the presence of azithromycin in growth-stimulating concentrations (6–8  $\mu\text{g/mL}$ ) slightly decreased (Fig. 8b).

This fact correlates with our hypothesis concerning biofilm growth stimulation in the presence of azithromycin, accompanied by increased synthesis of the DMMB-stained polysaccharide matrix, which, according to the literature [8], plays an important role in biofilm resistance to biocides and physicochemical stress factors. Additional evidence for this conclusion will be provided in the section of this study dedicated to microscopic research.

What is the probable biochemical mechanism of biofilm growth stimulation by azithromycin? Based on the literature, the following possible reasons for the stimulation of biofilm formation in pathogenic bacteria may be suggested.

First of all, enhanced biosynthesis of adhesion factors may occur, for example in the case of action of sub-inhibitory concentrations of the antibiotic imipenem on *Acinetobacter baumannii* [22]. Secondly, antibiotics may have a positive effect on the synthesis of QS regulation system factors required for biofilm formation, as was shown for macrolides in case of *C. violaceum* [23]. Additional evidence for importance of QS system in bacterial biofilm formation is provided by the work, in which another stress factor (hydrogen peroxide) was shown to stimulate growth of *P. aeruginosa* PAO1 biofilms. Moreover, this effect (similar to our experiments with azithromycin) was not observed in the presence of the plasmid pME6860, which contains the heterologous gene *aiaA* encoding *N*-acyl homoserine lactonase [24]. Finally, antibiotics may affect the regulatory systems involving cyclic diguanosine monophosphate (c-di-GMP), which participates in the biosynthesis of components of the polysaccharide biofilm matrix, as was shown for aminoglycosides in case of *E. coli* [25].

Thus, antibiotics not only induce death and growth inhibition of microorganisms, but are able to act as stress signatory molecules in sub-inhibitory concentrations [26].

**Effect of ultralow concentrations of azithromycin on biofilm formation by pseudomonads.** Investigation of the effect of a broad range of azithromycin concentrations on *P. chlororaphis* 449 revealed an unusual pattern: in regions of ultralow concentrations of the antibiotic which did not affect planktonic culture growth (0.001–0.01  $\mu\text{g/mL}$ ), biofilm formation was significantly inhibited (Fig. 9a).

Experiments with another strain, *P. chlororaphis* 66 confirmed this effect (Fig. 9b).

A similar effect was described in a recent work dedicated to detailed study of the inhibitory effect of macrolide antibiotics (erythromycin and azithromycin) on

stationary biofilms of *Porphyromonas gingivalis*, typical for dental plaque. Inhibitory effect on biofilms appeared at lower concentrations than the inhibitory concentrations for growth of planktonic cultures and for protein synthesis. Investigation of azithromycin-exposed biofilms revealed impaired matrix synthesis, while the amount of living cells remained the same [27].

It is still unclear how the same antibiotic can have different effects on the same bacterial biofilm, acting as an inhibitor at very low concentrations and as a stimulator at higher ones. Normally, a reverse pattern is common for toxic substances, i.e., stimulation at low concentrations and inhibition at high ones.

A possible explanation of this phenomenon is as follows. Biofilm matrix is a complex multi-component structure of biopolymers (DNA, proteins and polysaccharides). Since ultralow concentrations of azithromycin do not affect DNA and protein synthesis, we can suggest that in this case it targets mainly polysaccharide synthesis. Several polysaccharides take part in biofilm formation in pseudomonads. If synthesis of one of them is repressed by ultralow concentrations of azithromycin and synthesis of the second one is activated by higher concentrations of azithromycin, and if both processes are simultaneous, biofilms may be formed according to the variant shown on Fig. 9. At ultralow concentrations of azithromycin, biofilm growth is inhibited due to repressed synthesis of the first component, while synthesis of the second component has not been activated yet. At higher concentrations of azithromycin, activated synthesis of the second component compensates for the inhibition of the first component.

Undoubtedly, this hypothesis is speculative and lacks experimental proof.

**Phase contrast microscopy (PCM) and epifluorescent microscopy (EFM) application. PCM.** According to the literature, synthesis of the polysaccharides of the polymeric matrix is the most likely the target for azithromycin at both biofilm growth repression and biofilm stimulation. One of the methods making it possible to trace accumulation of the biofilm matrix polysaccharides is staining with DMMB, a specific stain for polysaccharides. In our previous work, we demonstrated that repression of the matrix polysaccharide synthesis (due to azithromycin activity or as a result of mutation) in some gram-negative bacteria correlates with decreased biofilm staining by DMMB [8].

Results shown on Figs. 10a–10c prove that matrix polysaccharides are the main target for repression or stimulation of biofilm growth by azithromycin, since the changes were more pronounced in DMMB-stained biofilms than in those stained with nonspecific CV.

The possible correlation of these conclusions was tested by microscopy of the biofilms exposed to various concentrations of azithromycin during their for-

mation and stained with the polysaccharide-specific stain DMMB.

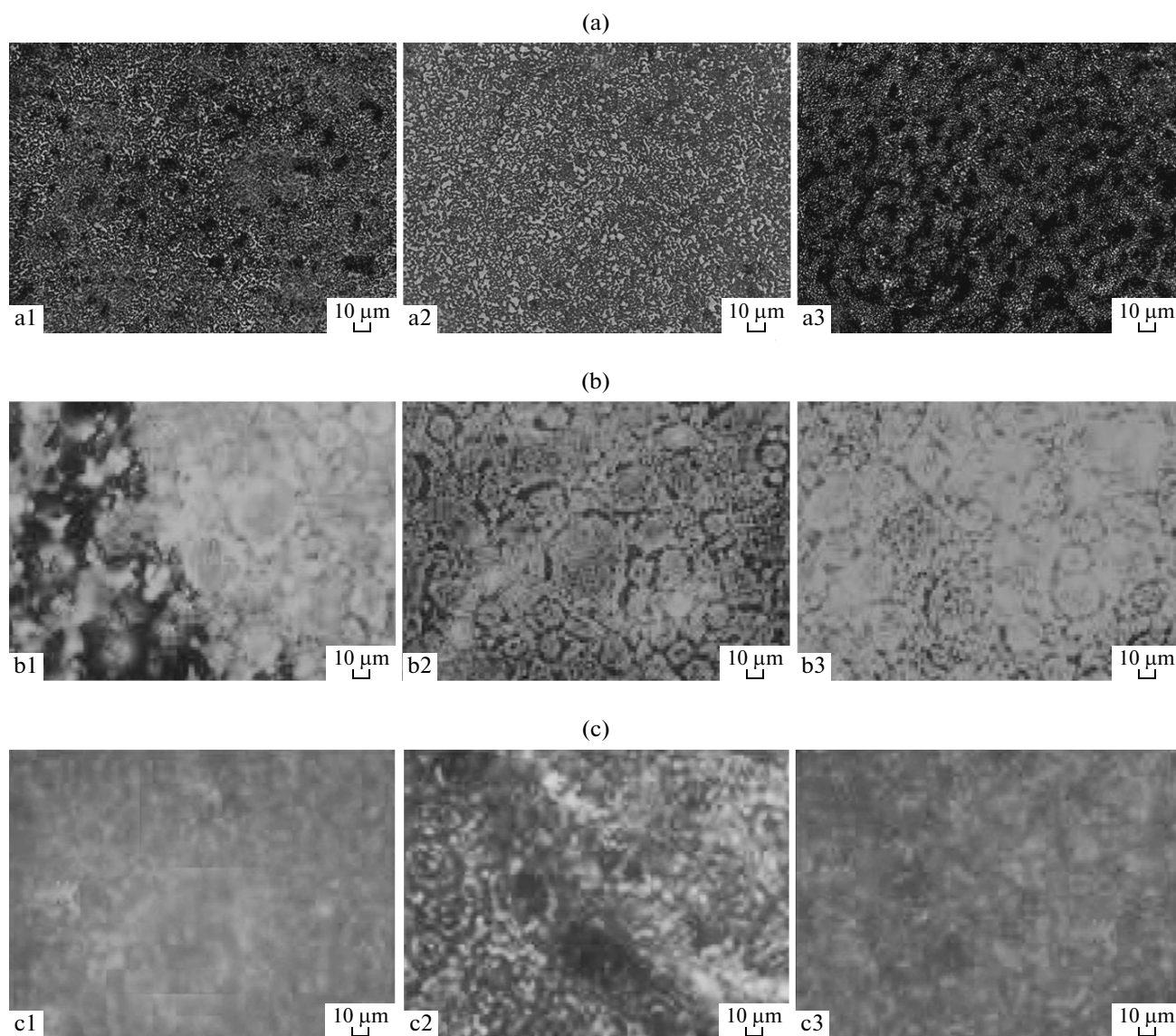
The results of phase contrast microscopy of *P. chlororaphis* 449 stained with DMMB after 6-h incubation (time required for matrix formation) in the presence of 0.03 (inhibitory concentration) and 8 µg/mL azithromycin (stimulating concentration) are shown on Fig. 10a. Azithromycin was not added to the control. Preliminary experiments demonstrated azithromycin not reacting with the stain and not influencing the staining process if added immediately before biofilm staining.

It can be seen that synthesis of the polysaccharide matrix components (stained with DMMB) decreased considerably in the presence of ultralow, “inhibiting” concentration of azithromycin (0.03 µg/mL), with a remarkable amount of cells becoming “naked.” On the contrary, in the presence of higher, “stimulating” concentrations of azithromycin (8 µg/mL) synthesis of the polysaccharide matrix components increased remarkably. These results confirm our suggestion of a dose-dependent regulatory effect of azithromycin on the synthesis of the polysaccharide matrix components.

**EFM.** The same incubation time (6 h) was used for biofilm incubation, which was sufficient for formation of a mature matrix. Since DAPI, apart from staining DNA, also exhibits nonspecific proper fluorescence, it is suitable for assessment of the total matrix content in biofilms. DAPI is accumulated in the matrix and visualizes it due to proper fluorescence of the unbound stain. Under these conditions, the fluorescence of matrix-embedded cells is masked [13]. Comparison of DAPI-stained biofilms in the control without azithromycin with the biofilms formed in the presence of “inhibitory” (0.03 µg/mL) and “stimulating” (8 µg/mL) concentrations of azithromycin demonstrated the total content of the matrix to decrease in the first case and to increase in the second case (Fig. 10b).

Since low concentrations of azithromycin affect neither growth of microbial cells nor protein and DNA synthesis, it may be assumed that decreased matrix content probably resulted from repression of polysaccharide synthesis.

The SYPRO Ruby dye stains proteins of the matrix associated with the cells and loosely located in the matrix. Comparison of the same variants of biofilms (as in Figs. 10a, 10b) stained with this stain (Fig. 10c) revealed the biofilm becomes looser in the presence of “inhibitory” azithromycin concentrations, with decreased protein content. According to previously expressed opinions, this decrease is probably due to impaired structure of the polysaccharide matrix, resulting in a loss of some proteins released into environment from the matrix. No significant changes in protein content in the biofilm were observed at “stimulating” concentrations of the antibiotic.



**Fig. 10.** Light microscopy of *P. chlororaphis* 449 biofilms grown in the presence of different concentrations of azithromycin: control without antibiotic (1), 0.03 µg/mL azithromycin (2), and 8.0 µg/mL azithromycin (3). PCM, staining with DMMB, dark areas correspond to the matrix (a); EFM, staining with DAPI, light areas correspond to the matrix (b); and EFM, staining with RUBY, light areas correspond to the matrix (c).

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Translated by E. Botchkova

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EXPERIMENTAL ARTICLES

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## Niclosamide as a Promising Antibiofilm Agent

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**Abstract**—Search for inexpensive efficient compounds with antibiofilm activity, which could be applied both for treatment of biofilm-associated infections and in other cases requiring biofilm elimination, is presently of importance. For this purpose we chose niclosamide. Niclosamide is a widespread and available anthelmintic drug, which was also recently shown to suppress bacterial growth. High antibiofilm activity of niclosamide against a range of gram-positive bacteria isolated from different ecological niches was shown. According to our results, niclosamide may be a promising component of composite antibiofilm preparations.

**Keywords:** biofilm, niclosamide, composite antibiofilm preparations

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The area of research that deals with physiological and biochemical features of microorganisms with a “sessile” lifestyle has recently marked its eightieth anniversary, assuming that it dates back to the seminal work of ZoBell (1935, 1937). He was the first to use an experimental model for monitoring the bacterial “bio-fouling” of surfaces. The term “biofilms” was applied to this phenomenon to describe the operation of bio-filters in wastewater treatment reactors, starting approximately from the 1960s (Nemchenko and Galushkina, 1978). However, this term became widely used in scientific literature after Costerton et al., had published their work in 1978. Even though a long period of time has elapsed, research on this cardinal issue has so far been concerned only with “the tip of the iceberg.” Moreover, different researchers come up with quite different terms to characterize the biofilm lifestyle of microorganisms, which is their predominant mode of life under natural conditions. We prefer to use the definition of the biofilm phenomenon that was suggested in our earlier review work (Nikolaev and Plakunov, 2007). In our opinion, it adequately describes the phenomenon: “biofilms represent spatially and metabolically structured communities of microorganisms that are embedded in the extracellular polymer matrix and are located at a phase boundary.” Importantly, this definition emphasizes the obligatory presence of the extracellular polymer matrix (EPM) that plays an important part in securing the resistance of biofilms to environmental factors.

Within biofilms, microorganisms carry out global biogeochemical processes. They are widely used in biotechnology, e.g., in wastewater treatment

(Nozhevnikova et al., 2015). They are still more important in medical terms, because pathogens as components of biofilms are much more resistant to extreme physical and chemical factors (Strelkova et al., 2013; Stewart et al., 2015), antibiotics (Strelkova et al., 2012; Balcázar et al., 2015), and the immune system of the host organism (De La Fuente-Núñez et al., 2013; Ribeiro et al., 2015).

According to the data of the Center for Disease Control and Prevention (CDC, United States), 65 to 80% of all pathogens are resistant to antibiotics (Hampton, 2013). With most pathogens, this is due to formation of microbial biofilms in the human organism (Venkatesan et al., 2015). Microbial biofilms were detected in 92% of patients with chronic otitis (Gu et al., 2014). With regard to intense ongoing adaptive mutagenesis, biofilms can serve as a reservoir in which antibiotic-resistant mutants and microbial persister cells accumulate (Plakunov et al., 2010; Conlon et al., 2015). “Collective” microbial resistance to antibiotics develops in a multispecies biofilm community (Vega and Gore, 2014).

Apart from endangering human health, microbial biofilms can develop in such man-made facilities as pipelines and water-contacting surfaces of various devices. This results in accelerating corrosion and making the devices inoperative (Xu et al., 2016). In light of all the above, researchers have to constantly pay attention to new methods of eliminating biofilms, including the screening for substances that inhibit biofilm formation. However, most of the antibiofilm agents described heretofore are toxic to animals and humans, which prevents their application to infection



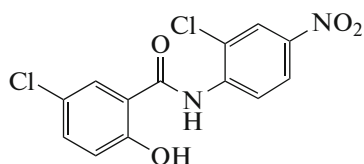


Fig. 1. The structural formula of niclosamide.

chemotherapy or necessitates the implementation of costly industrial production processes (Yang et al., 2014). In our work, a different approach is preferred that has been suggested recently. Traditional drugs or food additives that have been clinically tested and whose production is comparatively inexpensive, are screened for substances with antibiofilm activity (Imperi et al., 2013; Mart'yanov et al., 2014). We established earlier that 4-hexylresorcinol, a representative of the alkylhydroxybenzene class which is used as food preservative E586, exerts, in combination with the antibiotic azithromycin, a synergistic antimicrobial effect on the biofilms of a number of gram-positive and gram-negative bacteria (Mart'yanov et al., 2015).

Niclosamide, currently used as an anthelmintic and molluscicide (Jiang and Li, 2015), was chosen as the subject of this work. Recently, niclosamide has received considerably increased attention because of its prominent antitumor effect (Sack et al., 2011; Pan et al., 2012; Stewart et al., 2016). This effect is due to the fact that niclosamide inhibits a large number of signal pathways and is also capable of affecting mitochondria in such a way as to induce the apoptosis of malignant tumor cells (Moskaleva et al., 2015). Nevertheless, very little information is available in the literature on the antibacterial activity of niclosamide. A study was conducted that revealed an inhibitory influence of niclosamide on quorum-sensing systems in gram-negative bacteria (Imperi et al., 2013). There is only one report on the effect of niclosamide on biofilm formation in gram-positive bacteria (Torres et al., 2016).

The goal of the present work was to investigate the effect of niclosamide on biofilm formation in gram-positive bacteria that occupy various ecological niches (soil, the stratal waters of oil deposits, and the microbiota of the human skin) and to assess the prospective applications of niclosamide as a wide-spectrum antibiofilm agent. In addition, the feasibility of enhancing the antibiofilm effect by combining niclosamide with the antibiotic azithromycin was tested.

## MATERIALS AND METHODS

**Tested compounds.** Two biologically active substances were used in this work: the salicylanilide derivative of niclosamide (Ecochimtech, Russia) and the antibiotic azithromycin (OBL Pharm). The structural

formula of niclosamide (2',5-dichloro-4'-nitrosalicylanilide) is shown on Fig. 1. The drug is commercially known as phenasal, mansonyl, lintex, yomesan, phenodec, and phenalidon.

Niclosamide is poorly water-soluble. Therefore, its stock solution (5 mg/mL) was prepared in dimethyl sulfoxide (DMSO) (99.99%, ECOS-1, Russia; Paritex, Russia) and thereupon diluted with LB medium (Sigma) to obtain the desired concentration.

Azithromycin is a semisynthetic antibiotic that belongs to the macrolide class and contains a 15-atom lactone ring. It is obtained by inserting a nitrogen atom between carbon atoms 9 and 10 of the 14-atom lactone ring of macrolides (Maezono et al., 2011). Azithromycin suppresses protein synthesis by binding to the 50S ribosome subunit (Köhler et al., 2007). This substance is widely used against gram-positive and gram-negative bacteria as well as "atypical" pathogens (Gillis and Iglewski, 2004). Azithromycin exhibits some antibiofilm activity: it suppresses the growth of biofilms of *P. gingivalis* (Maezono et al., 2011) and *P. aeruginosa* (Bakheit et al., 2014). The stock solution of azithromycin was prepared in 96% ethanol and diluted with LB medium. It was established in special studies that, at the final concentrations used by us (below 0.1%), the aforementioned solvents affected neither the growth of planktonic cultures of the tested bacteria nor biofilm formation by them.

**Microorganisms.** In this work, strains of gram-positive bacteria (Table 1) from the collection of the Petroleum Microbiology Laboratory of the Biotechnology Federal Research Center, Russian Academy of Sciences were used. To determine the action spectrum of niclosamide, representatives of two ecological groups of microorganisms were used, human symbionts and saprotrophic bacteria from the stratal waters of oil deposits. Presumably, microorganisms from stratal waters have never contacted biocides in their environment. In contrast, human symbiotic strains that were isolated from the skin, identified, and used in this work, have been in contact with biocides.

**Identification of microorganisms.** DNA isolation was carried out with the Diatom DNA Prep 200 kit (Isogen, Russia) according to the manufacturer's recommendations. Amplification was performed with 20 ng of the genome DNA by means of the Phusion® High-Fidelity DNA Polymerase (NEB, United States). A commercially available working solution with all necessary components was used. To amplify the 16S rRNA gene, the primers 515-F: GTGCCAG-CMGCCGCGGTAA (Turner et al., 1999) and 907-R: CCGTCAATTCMTTTRAGTTT (Lane, 1991) were used. PCR was performed under the following conditions: at 95°C for 3 min; at 95°C for 15 s; at 58.8°C for 25 s; at 72°C for 30 s (35 rounds); and at 72°C for 5 min. Thereupon, the PCR mixture was purified on Agencourt AMPure XP granules (Beckman Coulter, United States). The fragments obtained

**Table 1.** Cultures of gram-positive bacteria used in this work

Name	Description	Source
<i>Staphylococcus aureus</i> MFP03	Gram-positive nonmotile facultatively anaerobic cocci. Human symbionts	Collection of the Laboratoire de microbiologie signaux et microenvironnement EA4312 Evreux, France. Isolated from the human skin
<i>S. aureus</i> 209P	Gram-positive facultatively anaerobic cocci. A laboratory strain	Collection of the Petroleum Microbiology Laboratory of the Winogradsky Institute of Microbiology, Biotechnology Federal Research Center, Russian Academy of Sciences
<i>Micrococcus luteus</i> C01	Gram-positive aerobic cocci that form tetrads. Colonies bright yellow. Human symbionts	Collection of the Petroleum Microbiology Laboratory of the Winogradsky Institute of Microbiology, Biotechnology Federal Research Center, Russian Academy of Sciences. Isolated from human skin.
<i>Kytococcus schroeteri</i> H01	Gram-positive aerobic cocci that form tetrads and aggregates. Colonies off-white. Human symbionts	Collection of the Petroleum Microbiology Laboratory of the Winogradsky Institute of Microbiology, Biotechnology Federal Research Center, Russian Academy of Sciences. Isolated from the human skin
<i>Dietzia natronolimnaea</i> 2610-1	Gram-positive aerobic rods. Hydrocarbons are metabolized	Collection of the Petroleum Microbiology Laboratory of the Winogradsky Institute of Microbiology, Biotechnology Federal Research Center, Russian Academy of Sciences. Isolated from the stratal waters of the Romashkino oil deposit
<i>Kocuria rhizophila</i> 4A-2Zh	Gram-positive aerobic rods that form aggregates in a liquid medium. Hydrocarbons are metabolized	Collection of the Petroleum Microbiology Laboratory of the Winogradsky Institute of Microbiology, Biotechnology Federal Research Center, Russian Academy of Sciences. Isolated from the stratal waters of the Romashkino oil deposit

were sequenced at the Genome Common Use Center (genome-centre.ru) on a 3730xl DNA Analyzer (ThermoFisher).

**Media and cultivation.** The microorganisms were stored at 4–5°C on semiliquid LB medium (Sigma) butts (5 mL) under paraffin oil. The inocula used in the experiments with biofilms were the 24-h cultures of *K. rhizophila* 4A-2Zh, *S. aureus* MFP03, *S. aureus* 209P, *Micrococcus luteus* C01, and *Kytococcus schroeteri* H01. They were cultivated in liquid LB medium on a shaker (150 rpm) at 30°C. *Dietzia natronolimnaea* 2610-1 was grown for 3 days under the same conditions in the LB medium supplemented with 0.5% of the mixture of liquid paraffins with 14–16 atoms in their carbon chains.

**Obtaining biofilms and assessing their growth.** The impact of niclosamide on biofilms was evaluated using the methods that were developed by us earlier (Plakunov et al., 2016). The first method is based on determining the number of metabolically active cells in biofilms grown on glass fiber micropore filters (Whatman®, GF/F, pore diameter 0.6–0.8 µm) that are placed on the surface of solid medium supplemented with niclosamide. The data are compared to

those obtained on the media without niclosamide. In these studies, 50 µL of diluted 24-h culture were applied to the filters. Dilution degree was optimized in special studies (Plakunov et al., 2016). The LB agar medium was used for *D. natronolimnaea* 2610-1, *M. luteus* C01, *K. schroeteri* H01, *K. rhizophila* 4A-2Zh, and *S. aureus* 209P; TSA medium (Carl Roth GmbH +Co) with 0.25% glucose was used for *S. aureus* MFP03. Metabolically active cells were detected with the dye 3-(4,5-dimethyl-2-il)-2,5-diphenyltetrazolium bromide (MTT, Sigma), an electron acceptor that is converted into water-insoluble blue formazan. Formazan was extracted with DMSO from biofilms, and optical density of the extract was measured thereupon with a KFK-2 MP photoelectric colorimeter (see below).

The second method was based on growing biofilms on the surface of Teflon cubes. It was used to monitor total biofilm growth and the content of metabolically active cells in the biofilms. Teflon cubes were placed in test tubes with liquid LB medium. After sterilization at 1 atm, the inoculum (50 µL of the culture grown as described above) was added. During incubation (20–24 h at 30°C, 150 rpm), biofilms formed on the cube

**Table 2.** Niclosamide ID<sub>50</sub> values for the tested strains in the studies with filters (MTT staining)

Strain	ID <sub>50</sub> , µg/mL
<i>S. aureus</i> MFP03	0.077
<i>S. aureus</i> 209P	0.05
<i>M. luteus</i> C01	0.067
<i>K. schroeteri</i> H01	0.054
<i>K. rhizophila</i> 4A-2Zh	0.174
<i>D. natronolimnaea</i> 2610-1	0.018

surface, and the planktonic culture developed in the culture liquid in the same test tube. Upon incubation, the planktonic culture was separated and its apparent optical density at 540 nm was determined. The remaining planktonic cells were removed by washing biofilm-covered cubes with 1% NaCl solution. The cubes were stained and rinsed with distilled water to remove the unbound dye. Either crystal violet (CV, Carl Roth GmbH +Co) or MTT was used as the dye. Biofilms-bound dye was extracted either with 96% ethanol (CV) or with DMSO (MTT). Thereupon, the optical density of the extract at 590 nm (CV) or within the 620–670 nm wavelength range (MTT) was measured and compared to that of bacteria-free control systems.

Staining biofilms with CV on filters was unfeasible because of the high level of nonspecific dye binding to the filter (Plakunov et al., 2016).

**Statistical treatment.** In this work, all results are represented as relative per cent values. The value of the apparent density of the cell suspension (for planktonic cultures) or of the dye extracted from biofilms without the inhibitor served as the control value that was assumed to be 100%. The optical density of experimental samples was expressed in per cent relative to the control value. This method of presenting data provided for quantitative comparison of the growth of planktonic cultures and biofilms during the same experiment.

The surface area of the cubes in each test tube equaled that of 8 wells of a standard 96-well plate, which provided for the validity of the results obtained.

Minimally, three filters were placed on the agar surface in each plate in biofilm cultivation experiments. After measuring the optical density of the extracts, the median optical density value was calculated for each experimental variant.

Statistical treatment of the results was performed using the OriginLab 8.6 software package. As the data in our experiment failed to conform with the normal data variation law, validity of the differences between results was estimated using the non-parametric Mann–Whitney–Wilcoxon test at  $p < 0.05$ . Graphic representation of the results obtained was based on the typical experiment selection algorithm (Paulson,

2008). Data plotting and smoothing was achieved with OriginLab 8.6 software by means of the B-spline or column plot functions.

## RESULTS AND DISCUSSION

Prior to the beginning of the work, human symbiotic strains were identified by analyzing their 16S rRNA gene fragments (the V4–V5 area). Strain C01 was similar to *M. luteus* C01 (99% 16S rRNA gene similarity), and H01 to *K. schroeteri*. Both strains are usual representatives of the skin microbiota, but *K. schroeteri* H01 is resistant to some antibiotics and can cause endocarditis in humans (Le Brun et al., 2005).

All microorganisms that were used in this work exhibited high sensitivity to niclosamide. This follows from the results given in Table 2.

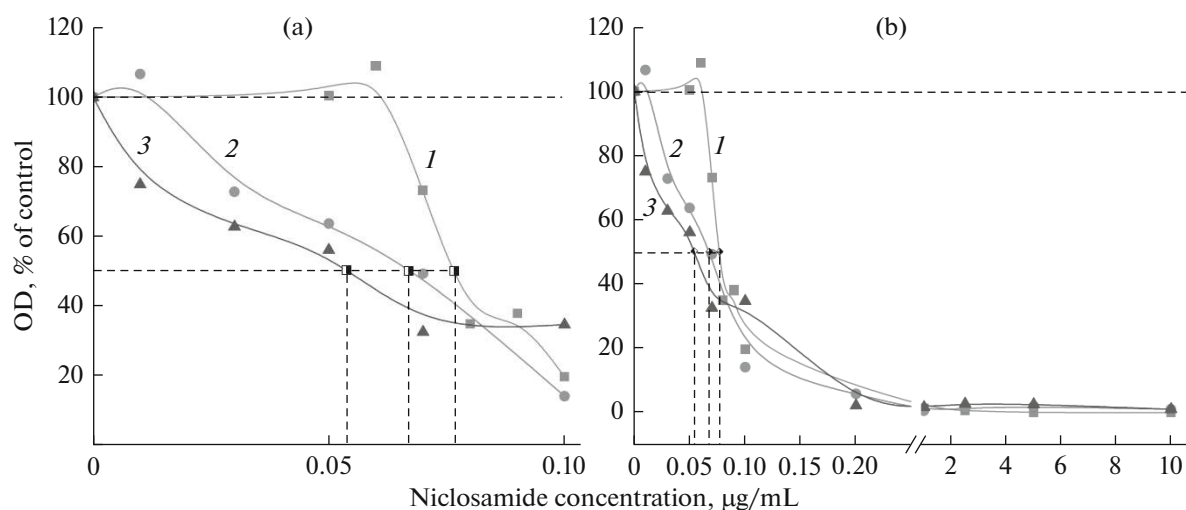
According to the data on biofilms grown on filters, human commensal strains exhibited similar sensitivity to niclosamide, i.e., they yielded similar ID<sub>50</sub> values (Fig. 2), i.e., biocide concentrations that inhibited microbial growth by 50%.

The stratal waters-isolated saprotrophs *D. natronolimnaea* 2610-1 and *K. rhizophila* 4A-2Zh differed in terms of resistance to niclosamide. Strain *D. natronolimnaea* 2610-1 revealed a higher sensitivity to niclosamide than the skin microbiota strains. Its ID<sub>50</sub> was 0.02 µg/mL, in contrast to 0.05 µg/mL in *S. aureus* 209P, the most sensitive strain among human symbionts (Fig. 3, Table 2). Presumably, this fact is due to the peculiarities of their habitats (ecological niches). Oil-oxidizing microorganisms deal with biocides more seldom than those that inhabit the human organism or its skin surface.

Niclosamide concentration of 0.1 µg/mL in the medium was sufficient to bring about a drastic decrease (up to 86% in *M. luteus* strain C01) in the number of metabolically active cells in the biofilm (Figs. 2b, 3). Under the influence of 1 µg/mL niclosamide, the number of metabolically active cells decreased almost to zero (to 0.5–1%, see Fig. 2) in *S. aureus* MFP03, *M. luteus* C01, *K. schroeteri* H01, and *D. natronolimnaea* 2610-1.

A diametrically opposite pattern was observed with *K. rhizophila* 4A-2Zh in studies with biofilms on MTT-stained filters. The culture exhibited a high resistance to niclosamide, in contrast to *D. natronolimnaea* 2610-1 and the commensal strains (Figs. 2, 3, and 4). The ID<sub>50</sub> value for *K. rhizophila* 4A-2Zh was 0.17 µg/mL, i.e., it was more than two times higher than that of *S. aureus* MFP03 (0.08 µg/mL).

In order to obtain still more precise data, biofilm formation by *K. rhizophila* 4A-2Zh on Teflon cubes was investigated (Fig. 5). The data obtained for *K. rhizophila* 4A-2Zh gives grounds for suggestions regarding the mechanism of the antibiofilm effect of niclosamide. In an analogy to the classical method



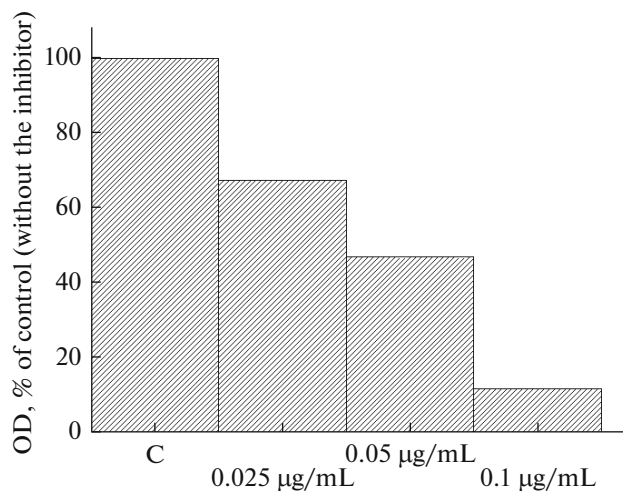
**Fig. 2.** Effect of niclosamide on the growth of biofilms in microorganisms from the skin microbiota. Cultivation on glass fiber filters (MTT staining): lower concentration range (a) and complete concentration range (b). Designations: *S. aureus* MFP03 (1), *M. luteus* C01 (2), and *K. schroeteri* H01 (3). The ID<sub>50</sub> point corresponds to the intersection between the perpendicular lines and the horizontal axis. Dashed line, control system without the inhibitor.

with immunological plates (Bakheit et al., 2014), the biofilm was in equilibrium with the planktonic culture in our studies with Teflon cubes. However, “forced adherence” was characteristic of the studies with filters: all microbial cells immediately adhered to filter fibers, whereas no planktonic culture developed.

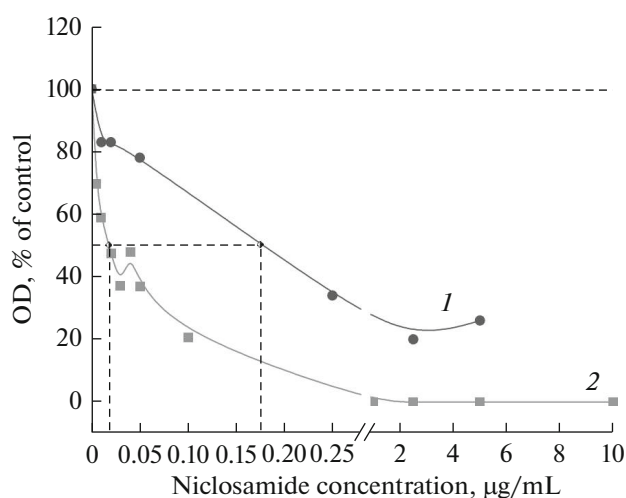
The ID<sub>50</sub> value (0.09 µg/mL) with biofilms growing on MTT-stained Teflon cubes was almost three times lower than that in the studies with filters. Nonetheless, the ID<sub>50</sub> values for planktonic cultures in these two systems were similar. Hence, the effect of niclosamide on the growth of biofilms that are in dynamic equilibrium with the planktonic culture is

more pronounced than without the planktonic culture. It follows that niclosamide primarily suppresses transition of bacterial cells to the attached mode of life.

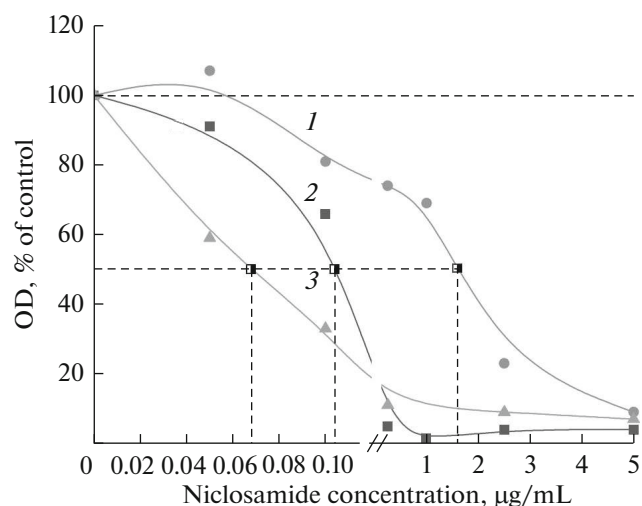
If biofilms were stained with CV, the ID<sub>50</sub> value was 1.6 µg/mL, which exceeded the ID<sub>50</sub> in the MTT-stained system by two orders of magnitude. Based on these data, the following hypothesis was advanced. *K. rhizophila* 4A-2Zh is characterized by a very strong tendency to form aggregates in liquid medium. The aggregates are in fact biofilms that are very difficult to separate into individual cells. It should be noted that



**Fig. 3.** Effect of niclosamide on the growth of *S. aureus* 209P biofilms. Cultivation on MTT-stained glass fiber filters.



**Fig. 4.** Effect of niclosamide on the growth of biofilms of oil-oxidizing microorganisms: *K. rhizophila* 4A-2Zh (1) and *D. natronolimnaea* 2610-1 (2). Cultivation on MTT-stained glass fiber filters. ID<sub>50</sub> determination as on Fig. 2.



**Fig. 5.** Effect of niclosamide on the growth of *K. rhizophila* 4A-2Zh. Cultivation of biofilms on Teflon cubes. CV-stained biofilms (1), planktonic culture (2), and MTT-stained biofilms (3). Designations are as on Fig. 2.

the inoculum for the studies with filters and cubes was tested for the presence of aggregates, and only a homogenous planktonic cultures without aggregates was used. The properties of CV that is capable of non-specifically staining both the matrix and the cells in the biofilm give grounds for the suggestion that a culture with such high biofilm-forming activity is unlikely to lose its matrix-synthesizing capacity even in the presence of a substance that affects the adhesion process.

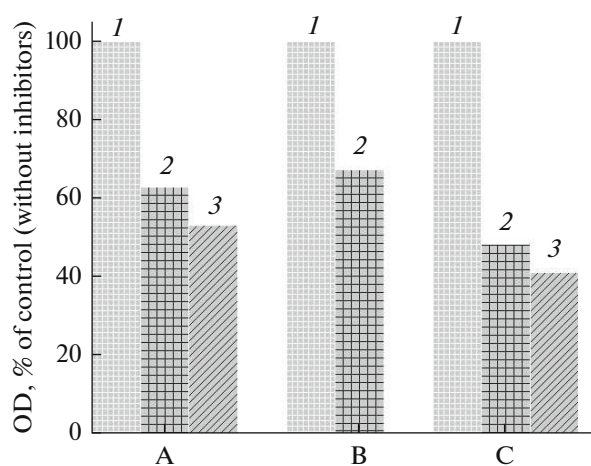
*K. rhizophila* 4A-2Zh growth resumed and reached the control level (data not shown) after transferring the filter from the surface of LB agar with 5 µg/mL niclosamide to the surface of LB medium without it. This provides evidence for the bacteriostatic mode of action of niclosamide.

For *S. aureus* 209P and *K. rhizophila* 4A-2Zh, potential applicability of niclosamide as a component of binary antibiofilm preparations in combination with the antibiotic azithromycin was shown (Figs. 6 and 7). The azithromycin and niclosamide concentrations were adjusted in order to successfully monitor the combined effect. Each of the substances per se suppressed biofilm formation by 30–60%.

A weak additive effect was observed when niclosamide at a concentration of 0.025 µg/mL was combined with azithromycin.

A still more pronounced inhibitory effect was produced by the niclosamide–azithromycin combination on the biofilms of *K. rhizophila* 4A-2Zh (Fig. 7).

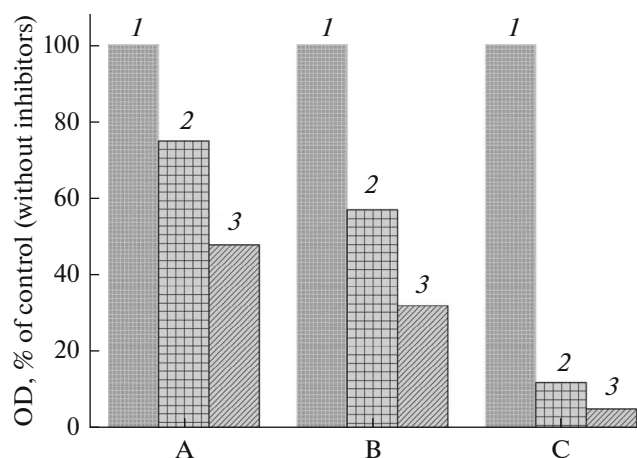
Thus, our studies revealed the efficiency of niclosamide as an antibiofilm agent with a broad action spectrum. It produces an effect on microorganisms from various ecotopes, which are exemplified in



**Fig. 6.** Effect of azithromycin alone and in combination with 0.025 µg/mL niclosamide on biofilm formation by *S. aureus* 209 P on MTT-stained glass fiber filters. Control system without inhibitors (1). A: azithromycin, 0.25 µg/mL (2) and azithromycin, 0.5 µg/mL (3). B: niclosamide, 0.025 µg/mL (2). C: niclosamide, 0.025 µg/mL + azithromycin, 0.25 µg/mL (2) and niclosamide, 0.025 µg/mL + azithromycin, 0.5 µg/mL (3).

the present work by microbiota of human skin and of the stratal waters of oil deposits.

The results obtained enable us to consider niclosamide as a promising component of composite antibiofilm preparations (in combination with antibiotics). First, large quantities of niclosamide are produced around the world, including Russia, which



**Fig. 7.** Effect of niclosamide and azythromycin (applied separately and in combination) on biofilm formation by *K. rhizophila* 4A-2Zh on MTT-stained glass fiber filters. Control system without inhibitors (1). A: azithromycin, 0.01 µg/mL (2) and azithromycin, 0.05 µg/mL (3). B: niclosamide, 0.1 µg/mL (2) and niclosamide, 0.25 µg/mL (3). C: niclosamide, 0.1 µg/mL + azithromycin, 0.01 µg/mL (2) and niclosamide, 0.1 µg/mL + azithromycin, 0.05 µg/mL (3).



could facilitate the development of composite antibiofilm preparations. Second, niclosamide is relatively inexpensive. Third, it is characterized by very high activity: several dozens of µg/mL of niclosamide decrease the number of metabolically active cells by 50%. Fourth, niclosamide is not toxic to the host organism at tested concentrations.

The additive mode of interaction of niclosamide and azithromycin can be due to the different mechanisms of their antibiotic activities. Azithromycin suppresses protein synthesis at the translation stage by interacting with the 50S ribosome subunit (Bakheit et al., 2014). Accordingly, suppression of biofilm formation can result from a lack of necessary enzymes. As noted above, niclosamide was revealed to produce an inhibitory effect on the QS system of *P. aeruginosa* by suppressing *N*-acylhomoserine lactone synthesis (Imperi et al., 2013). However, niclosamide also exerts an antibiofilm effect against gram-positive bacteria that possess fundamentally different QS systems. According to our results obtained, niclosamide affects the processes that are involved in the transition of planktonic cells to the attached mode of life. Nevertheless, the molecular mechanisms of action of niclosamide on biofilm formation remain to be addressed in future research.

#### ACKNOWLEDGMENTS

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Translated by A. Oleskin

# Effect of two cosmetic compounds on the growth, biofilm formation activity, and surface properties of acneic strains of *Cutibacterium acnes* and *Staphylococcus aureus*

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## Abstract

Increasing popularity of preservative-free cosmetics necessitates in-depth research, specifically as bacteria can react to local factors by important metabolic changes. In this respect, investigating the effect of cosmetic preparations on pathogenic strains of commensal species such as acneic forms of *Cutibacterium acnes* (former *Propionibacterium acnes*) and bacteria behaving both as commensals and opportunistic pathogens such as *Staphylococcus aureus* is of major interest. In this study, we studied the effect of commonly used cosmetics, Uriage<sup>™</sup> thermal water (UTW) and a rhamnose-rich polysaccharide (PS291<sup>®</sup>) on RT4 and RT5 acneic strains of *C. acnes* and a cutaneous strain of *S. aureus*. UTW affected the growth kinetic of acneic *C. acnes* essentially by increasing its generation time and reducing its biomass, whereas only the *S. aureus* final biomass was decreased. PS291 had more marginal effects. Both compounds showed a marked antibiofilm activity on *C. acnes* and *S. aureus*. For *S. aureus* that appeared essentially due to inhibition of initial adhesion. Cosmetics did not modify the metabolic activity of bacteria. Both *C. acnes* and *S. aureus* showed marked hydrophobic surface properties. UTW and PS291 had limited effect on *C. acnes* but increased the hydrophobic character of *S. aureus*. This work underlines the effect of cosmetics on cutaneous bacteria and the potential limitations of preservative-free products.

## KEYWORDS

biofilm, cosmetics, *Cutibacterium acnes*, metabolism, *Staphylococcus aureus*, surface adhesion, surface polarity

## 1 | INTRODUCTION

Human skin is the largest human organ, which harbors hundreds of microbial genera (Grice et al., 2009). This coexistence developed in the course of evolution: while the host organism does not prevent growth of commensal microorganisms, it attacks pathogenic

invaders (sometimes with the aid of commensals) (Chiller, Selkin, & Murakawa, 2001). Species ratios of microorganisms on human skin depends on sex, age, geographic region, etc., although the patterns within a group are almost the same (Grice et al., 2009; San Miguel & Grice, 2014). Microenvironment determined by the physiological properties of skin is among the most important factors affecting

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microbial growth. Three types of physiological environment, greasy, moist, and dry, may be discerned depending on position of a skin sample and localization of sweat and oil glands (Grice et al., 2009). Each type is characterized by different ratios of microbial species. Actinobacteria (particularly *Cutibacteria*) and Firmicutes (essentially *Staphylococci*) prevail at greasy and moist sites, while Betaproteobacteria and Corynebacteria predominate at dry ones (Grice et al., 2009; San Miguel & Grice, 2014).

*Cutibacterium acnes* (former *Propionibacterium acnes*) (Scholz & Kilian, 2016) is the most common member of the genus associated to the human skins (Bruggemann, 2004). These are gram-positive diphtheroid aerotolerant rod-shaped anaerobic microorganisms (Aubin, Portillo, Trampuz, & Corvec, 2014). *C. acnes* is a component of the normal microbiota, an opportunistic pathogen preferring anoxic, fat-rich niches, such as sebaceous glands (Aubin et al., 2014; Bruggemann, 2004; Fitz-Gibbon et al., 2013). *C. acnes* is traditionally considered involved in the pathogenesis of many cases of acne, one of the most common skin disorders, although direct evidence is still lacking (Achermann, Goldstein, Coenye, & Shirliff, 2014; Fitz-Gibbon et al., 2013). In this regard, *C. acnes* biofilms, found inside sebaceous glands, are considered among a possible major causes of acne (Coenye, Peeters, & Nelis, 2007) and, as these biofilms provide high resistance to antimicrobial agents and to the action of the host immune system (Achermann et al., 2014; Coenye et al., 2007; Nikolaev & Plakunov, 2007; Nozhevnikova, Botchkova, & Plakunov, 2015), they play a critical role *C. acnes* in the pathogenicity. *C. acnes* is also a species known for its high heterogeneity and it was divided into four phylogenetic groups (IA, IB, II and III), group IA being associated to the more severe forms of acne (McDowell et al., 2010). More recently, the collection, ribotyping, and genome sequencing of *C. acnes* on normal skin and on the skin of volunteers suffering of diverse degree of acne, revealed that some principal ribotypes bearing a specific locus (Locus 3) of plasmidic origin are associated to acne (Fitz-Gibbon et al., 2013). Environmental parameters, and particularly cosmetic compounds, should be also modulating the biofilm formation activity and virulence of these microorganisms (Enault, Saguet, Yvergniaux, & Feuilloley, 2014) but until now these studies were never realized on acneic strains of *C. acnes*. In its ecological niche, such as the hair follicle, *C. acnes* is associated to another lipophilic microorganism, *Staphylococcus aureus* (Frenard et al., 2018), a gram-positive facultative anaerobe belonging to the phylum Firmicutes. *S. aureus* is a component of the normal skin microbiome (Aryee & Edgeworth, 2017) although it can also behave as an opportunistic pathogen and recent studies suggest that *C. acnes* should participate to *S. aureus* skin colonization and biofilm formation (Tyner, Patel, Tyner, & Patel, 2016).

Considering the high heterogeneity of *C. acnes* (McDowell et al., 2010) it appeared essential to fully document the biofilm formation activity and response to cosmetic compounds of the more critical of those bacteria associated to acne, such as RT4 and RT5 strains which bear a virulence plasmid associated to the more severe forms of acne (Fitz-Gibbon et al., 2013). In the present work we investigated the effect of Uriage™ thermal water (UTW) and rhamnose-rich

polysaccharide (PS291®). UTW is a natural isotonic mineral solution from an Alpine spring and is often used for face care (Uriage, 2018). PS291, also designated as Teflose for its coating activity (Solabia Biotecnologica, 2018) is a branched polysaccharide containing rhamnose, glucuronic acid, and glucose. Both compounds enter into the formulation of cosmetic products designed for sensitive skin, mild to moderate acneic skin, as well as in the used in deodorants or antiperspirants. The action of these compounds on *C. acnes* was compared to that on a strain of *S. aureus*, MFP03, collected on human skin exempt of any clinical sign (Hillion et al., 2013).

## 2 | MATERIALS AND METHODS

### 2.1 | Bacterial strains and culture

*Cutibacterium* (former *Propionibacterium* [4]) *acnes* acneic strains ribotype 4 (RT4) HLO45PA1 and ribotype 5 (RT5) HLO43PA2 initially isolated by Fitz-Gibbon (Fitz-Gibbon et al., 2013) were obtained from BEI Resources American Type Culture Collection (Virginia, United States). These strains associated to severe forms of acne differ from nonacneic strains (ribotype 6 and in a lower extend ribotypes 1, 2, and 3) by a large plasmide (Locus3) which should confer their virulence properties (Fitz-Gibbon et al., 2013). Bacteria stored at  $-80^{\circ}\text{C}$  were initially plated on agar brain heart infusion (BD). As these strains are strictly anaerobic, the plates were incubated for 72 hr in BD GasPack™ under anoxic conditions at  $37^{\circ}\text{C}$ . Colonies were then transferred into sterile conical 15 ml tubes (Falcon) filled to maximal capacity with reinforced clostridial medium (RCM) and grown for 72 hr at  $37^{\circ}\text{C}$ . For all experiments *C. acnes* was grown in RCM.

*S. aureus* MFP03 was initially isolated from the skin of healthy volunteers (Hillion et al., 2013). It was characterized by phenotypic, metabolic, 16sRNA sequencing and MALDI-biotyper total proteome analysis. For culture, an aliquot of bacteria stored at  $-80^{\circ}\text{C}$  ( $\pm 0.5$  mg) was mixed in 3 ml of the Luria-Bertani (LB) broth and incubated for 24 hr at  $37^{\circ}\text{C}$  under agitation. For microcolony formation in dynamic conditions and adhesion to solvents *S. aureus* MFP03 was grown in tryptone-soy broth (Biocar Diagnostics) with 0.25% glucose.

### 2.2 | Tested compounds

UTW is a natural isotonic water ( $\text{pH} \approx 7.0$ ) containing approximately 11 g/L salts. Its mineral composition (g/L) is: sulfates, 2.86; chlorides, 3.5; and bicarbonates, 0.39. Elemental composition is as follows: sodium, 3.5 g/L; calcium, 0.6 g/L; magnesium, 0.125 g/L; potassium, 0.0455 g/L; silicon, 0.042 g/L; zinc, 0.16 mg/L; manganese, 0.154 mg/L; copper, 0.075 mg/L; and iron, 0.0015 mg/L. The water is stored in sterile conditions at room temperature. Controls were realized by use of physiological water ( $\text{NaCl}$  0.9%  $\text{pH} = 7.0$ —PS). PS291® is a rhamnose-rich polysaccharide obtained by fermentation and patented by BioEurope. It was used as a saturated solution in a water-propanediol mixture. These compounds were selected in coherence with previous studies labeled by the French

national cluster "Cosmetic Valley" (Enault et al., 2014; Mijouin et al., 2013) and CNRS GDR Cosm'Activ. ETU and PS291 were tested at 50% and 4%, respectively, corresponding to the concentrations potentially present on skin during exposure to cosmetics. As described in the result chapter, the effect of ETU on bacteria was compared to that observed using a same percentage of PS or complete RCM. For studies realized on PS291 the control medium was supplemented with a same amount of water-propanediol mixture.

### 2.3 | Bacterial growth kinetic

Effect of UTW and PS291 on the growth of *C. acnes* and *S. aureus* was studied in the F system SAFAS spectrophotometer incubators (Flex-Xenius XM; SAFAS, Monaco). The SAFAS system uses 96-well flat-bottomed polystyrene plates (NUNC). The inoculum was added to an initial  $OD_{580} = 0.08$ – $0.1$ . In the case of *C. acnes*, peripheral wells were filled with a  $CO_2$ -producing solution and prepared in anoxic conditions using GasPack™ system and sealed with Parafilm before incubation. Bacteria were incubated at 37°C for 72 hr (*C. acnes*) or 24 hr (*S. aureus*). Optical density of the cultures was determined automatically every 15 min. Growth curves were determined over a minimum of three independent experiments. Statistical variability was minimal and error bars (SEM) do not appear in the figure.

### 2.4 | Biofilm investigation using crystal violet staining

Biofilms were grown in NUNC. Experiments were carried out according to the modified classical procedure (O'Toole, 2011). The mixture (200 µl) of the medium and solutions of tested compounds was dispensed into the wells, and inoculum with  $OD_{580} = 1.7 \pm 0.2$  was added. The ratio of inoculum and medium volumes for *S. aureus* and *C. acnes* were 1:40 and 1:6, respectively. The plates were incubated at 37°C: 24 hr on the shaker for *S. aureus* and under static conditions in a GasPack™ system for 72 hr for *C. acnes*. Planktonic cultures were then removed, and the wells were washed thrice with PS to remove remaining planktonic cells. The plates were then dried for 5–10 min at 60°C, and the biofilms were fixed with 96% ethanol for 15 min. After fixation, ethanol was removed, the plates were dried and stained with 0.1% crystal violet (CV) for 15 min. After staining, the plates were washed with water for complete removal of dissolved CV. Biofilm-bound CV was extracted with 96% for 60 min, and optical density of the extract was measured at  $\lambda = 595$  nm on a Biorad spectrophotometer. Four wells were used for every sample. The experiments were carried out in at least three replicates. The results are expressed as  $M \pm SEM$  and statistical differences were determined using the Mann–Whitney nonparametric criterion. Graph plotting and calculation were carried out using Microsoft Excel 2007. The differences between experimental and control variants were considered reliable at confidence coefficient >95% ( $p < 0.05$ ).

### 2.5 | Confocal laser scanning microscopy

Observations were realized using an LSM 710 inverted confocal laser-scanning microscope (Zeiss, Germany). Three-dimensional (3D) images and orthocuts were obtained using Zen® 2009 software. Biofilm thickness was quantified with the same software. For these studies bacteria were grown in 24-well plate with flat glass bottom (Sensoplate, Geiner bio-one, Germany). Biofilms were grown according to Coenye (Coenye et al., 2007) with modifications. *C. acnes* cultures in RCM (72-hr old) were collected by centrifugation, washed twice with sterile PS, and resuspended in sterile PS at  $OD_{580} = 1$ . The suspension (300 µl) was applied to the wells and incubated for 2 hr at room temperature under anoxic conditions. The suspension was then removed, and the wells were washed twice with sterile PS. One well was fixed for subsequent visualization of primary bacterial adhesion. The medium (1 ml) supplemented with tested substances was then dispensed into the wells. The plates were incubated for 72 hr under static conditions at 37°C. The same technique was used for *S. aureus* except that the culture was realized in normal atmosphere and avec 24°C.

For visualization, the biofilms were stained with SYTO9 Green and subsequently treated with ProLong® Diamond Antifade Mountant (Molecular Probes™). Images are representative of the biofilm structure observed in a mean of 20 different fields over a minimum of four independent studies. Biofilm density and thickness were calculated over the same number of observations using Zeiss Zen Image Analysis software for light microscopy (ImageJ software package). Statistical differences were determined using the Mann–Whitney nonparametric criterion. The differences between experimental and control variants were considered reliable at confidence coefficient >95% ( $p < 0.05$ ).

### 2.6 | Study of microcolony formation in dynamic conditions

In order to differentiate potential effects on the initial adhesion step of *S. aureus*, an original technique of microcolony formation in dynamic conditions was employed. In this case, 1 ml of medium supplemented with the tested substances was dispensed into the wells and inoculated (1:40) with the bacterial strain. No adsorption phase was allowed and the plate was immediately incubated on a shaker at 180 rpm for 24 hr at 37°C. In this condition, only a subset of the more adherent bacteria can interact with the surface and form microcolonies into the wells. These microcolonies were stained with SYTO9 Green, as previously described, and observed using the LSM 710 inverted confocal laser scanning microscope. Images are representative of microstructure observed in a mean of 20 different fields over a minimum of three independent studies. The results are expressed as  $M \pm SEM$  and statistical differences were determined using the Mann–Whitney nonparametric criterion. Graphs were calculated using Microsoft Excel 2007. The differences between experimental and control variants were considered reliable at confidence coefficient >95% ( $p < 0.05$ ).

## 2.7 | Investigation of bacterial metabolic activity

The potential effect of UTW and PS291 on the bacterial metabolic activity in biofilms was determined as described by Plakunov (Plakunov, Mart'yanov, Teteneva, & Zhurina, 2016). The method is based on biofilm staining with 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT). MTT is used to reveal viable, metabolically active cells, acting as an electron acceptor for the electron transport chain; as a result, it is converted to water-insoluble, blue formazan (Berridge & Tan, 1993). The biofilms were grown on GF/F glass fiber filters (Whatman®) in Petri dishes with agar medium. *C. acnes* RT4 and RT5 were grown using solid RCM medium. Tryptone-soy agar with 0.25% glucose was used for *S. aureus* MFP03. Sterile filters (2 × 2 cm) were placed on the agar, and the culture (50 µl) was applied to the center. OD<sub>540</sub> values for *C. acnes* and *S. aureus* were 1.0 and 0.1, respectively. The biofilms were grown at 37°C for 72 hr (*C. acnes*) or 24 hr (*S. aureus*). After incubation the biofilm-bearing filters were stained with 0.1% MTT in sterile LB for 20 min, washed with distilled water, and dried. Formazan was then extracted with dimethyl sulfoxide (DMSO, EKOS-1, Russia; Pariteks, Russia). Optical density of the extract characterizing the number of metabolically active cells in the biofilm was measured at  $\lambda = 590$  nm. All experiments were carried out in at least three replicates. The data were analyzed using the Mann-Whitney nonparametric criterion. The differences between experimental and control variants were considered reliable at confidence coefficient >95% ( $p < 0.05$ ).

## 2.8 | Characterization of bacterial surface polarity

Cultures of *C. acnes* and *S. aureus* in stationary phase were harvested (7,000 × g; 10 min) and washed twice in PS. The polarity of control and treated bacteria was studied using the Microbial Adhesion to Solvent (MATS) technique (Bellon-Fontaine, Rault, & van Oss, 1996) and four solvents: chloroform, hexadecane, ethyl acetate, and n-decane. Briefly, bacterial cultures in early stationary phase and growth in the presence of ETU 50%, PS291® or the respective control medium, were harvested by centrifugation at 10,000 × g. Pellets were rinsed 2 times with NaCl 0.9% in water and diluted to OD<sub>400</sub> 0.8. An aliquot of bacterial suspension (2.6 ml) at was mixed for 60 s with 0.4 ml of each of the solvents indicated above. The tubes were vigorously hand-shaken and the OD<sub>400</sub> of the aqueous phase was measured after 15 min of decantation. In these conditions, bacteria are distributing between the water and solvent phases following their surface hydrophobic or hydrophilic character and Lewis acid/base balance (electronegative or electropositive character). The OD of the aqueous phase was measured at 400 nm. The percentage of cells in each solvent was calculated using the following equation:  $(1 - A/A_0) \times 100$ . The experiments were carried out in at least five replicates. The data were analyzed using the Mann-Whitney nonparametric criterion. The differences between experimental and control variants were considered reliable at confidence coefficient >95% ( $p < 0.05$ ).

## 3 | RESULTS

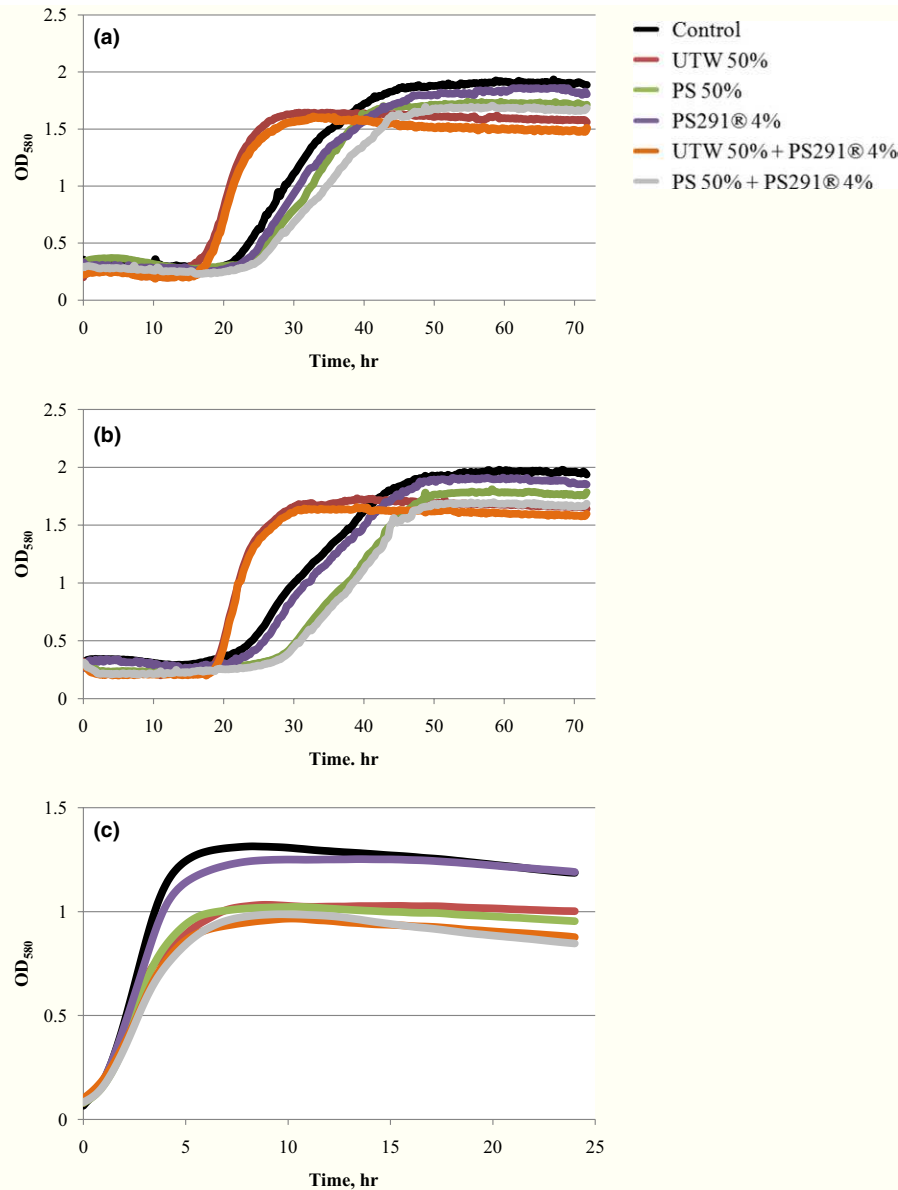
### 3.1 | Effect of thermal water and PS291® on the growth kinetics of acneic strains of *C. acnes* and *S. aureus*

Both acneic strains of *C. acnes* had a general similar behavior when they were exposed to UTW or PS291®. In the case of the RT4 strain, addition of PS291 was only associated to a minor shift of the growth kinetic but the slope of the curve (generation time) and the plateau (biomass) were almost identical (Figure 1a). In contrast, UTW 50% markedly reduced the lag time of the *C. acnes* growth curve with a 50% OD<sub>max</sub> that was reached after only 30 hr in the presence of UTW whereas it required 45 hr in the control study. Conversely the OD<sub>max</sub> of the bacterial grown in the presence of ETU was reduced suggesting a decrease in biomass formation. The reaction of the RT5 strain of *C. acnes* to PS291 was different with an apparent increase of the mean generation time from 0500 to 0530 hr, whereas the final OD and biomass was identical (Figure 1b). As observed with the R4 strain, UTW also reduced the lag time (from 44 hr in the control to 29 hr for UTW treated bacteria).

As observed with *C. acnes*, when *S. aureus* MFP03 was exposed to PS291 4% only minor changes in the growth kinetic were observed (Figure 1c). Conversely, UTW 50% induced a marked decrease of the OD<sub>max</sub> ( $M = -23\%$ ) whereas the generation time appeared unchanged. PS 50% had identical effects with a reduction of the final biomass without noticeable change of the generation time. The behavior of *S. aureus* in the presence of UTW 50% + PS291 4% was identical to that observed using UTW alone. By the same way, PS 50% + PS291 was leading to a reduction of the total biomass identical to that observed using PS 50% alone.

### 3.2 | Effect of thermal water and PS291® on the biofilm formation activity of acneic strains of *C. acnes* and *S. aureus*

The study was realized using the crystal violet technique which allows a straining of both bacteria and biofilm extracellular matrix. As shown in Figure 2, UTW 50% and PS291 4% had a strong effect on the biofilm formation activity of *C. acnes* RT4 and RT5 strains that was reduced to  $30.8 \pm 17.4$  and  $59.5 \pm 23.6\%$  of the control with UTW 50% and  $70.9 \pm 16.7$  and  $41.2 \pm 21.8\%$  of the control with PS291 4%. PS 50% also reduced the biofilm formation of *C. acnes* RT4 and RT5 to  $78.9 \pm 33.6$  and  $65.7 \pm 6.7$  of the control. As observed in growth kinetic studies, no visible additive effect of UTW with PS291 was observed when bacteria were exposed to the two factors in association. In the presence of UTW 50% and PS291® 4%, the biofilm formation activity of the strains of *C. acnes* RT4, RT5, and *S. aureus* reached  $50.5 \pm 13.5$ ,  $58.7 \pm 10.3$ , and  $25.4 \pm 6.5\%$  of the control, respectively. However, the inhibitory effect of the association of UTW 50% with PS291 4% was stronger than when PS291 was associated to PS 50%. In this case, the biofilm formation activity of *C. acnes* RT4, RT5, and *S. aureus* reached only  $86.1 \pm 11.1$ ,  $72.6 \pm 16.8$ , and  $60.7 \pm 12.5$  of the control, respectively.

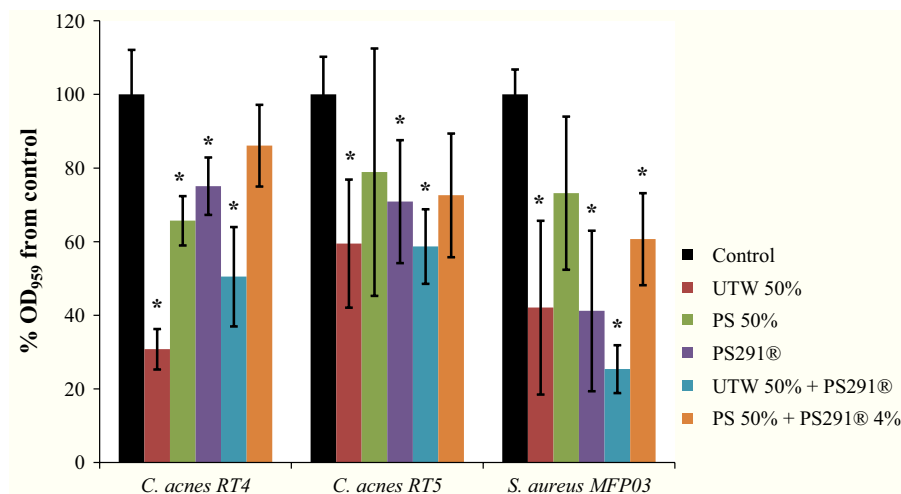


**FIGURE 1** Effect of thermal water and rhamnose-rich polysaccharide (PS291®) on the growth kinetics of acneic strains of *Cutibacterium acnes* and *Staphylococcus aureus*. (a) Effect of Uriage thermal water (UTW) and PS291® on the growth kinetics of acneic strain of *C. acnes* RT4. (b) Effect of UTW and PS291® on the growth kinetics of acneic strain of *C. acnes* RT5. (c) Effect of UTW and PS291® alone or in association on the growth kinetics a cutaneous strain of *S. aureus* (MFP03). Physiological water (PS; NaCl 0.9%) and medium supplement with 4% water-propanediol mixture were used as controls of UTW and PS291®, respectively. Curves are the mean of three independent experiments. Statistical variability was minimal and error bars (SEM) do not appear in the figure

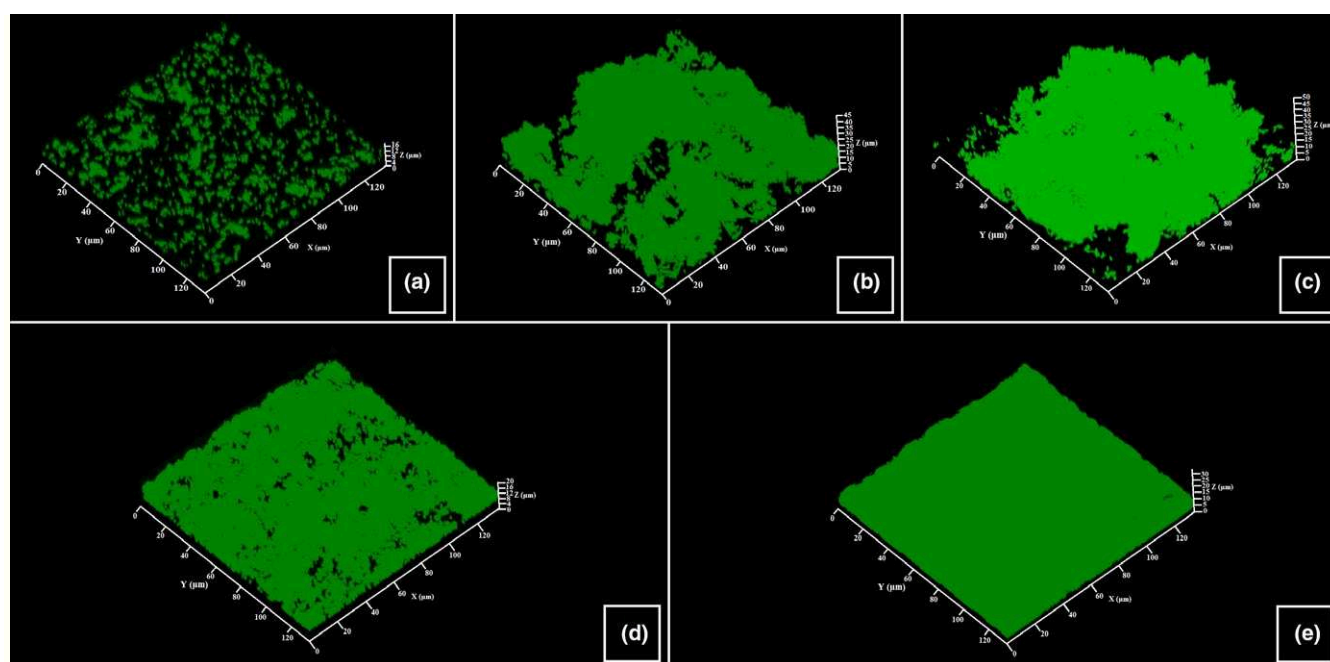
The structure, thickness, and biomass density of the biofilms formed by *C. acnes* RT4, RT5, and *S. aureus* MFP03 in the presence of UTW and PS291 was investigated by confocal laser scanning microscopy and image analysis. Using the *C. acnes* RT4 we observed a partial biofilm formation with 2 hr that was corresponding to the initial adhesion step (Figure 3a). A mature biofilm (mean thickness =  $35.3 \pm 6.2 \mu\text{m}$ /density =  $30.3 \pm 6.4 \mu\text{m}^3/\mu\text{m}^2$ ) was obtained after 72 hr of incubation (Figure 3b). When *C. acnes* RT4 was grown in the presence of PS 50% the mean thickness and biofilm density were not significantly modified (thickness =  $32.6 \pm 5.1 \mu\text{m}$ /density =  $28.7 \pm 4.7 \mu\text{m}^3/\mu\text{m}^2$ ) (Figure 3c). UTW 50% had a more

visible effect, with a reduction of biofilm thickness and density to  $24.9 \pm 4.4 \mu\text{m}$  (~29.4%) and  $21.7 \pm 4.1 \mu\text{m}^3/\mu\text{m}^2$  (~28.3%), respectively, (Figure 3d). PS291 4% also decreased the mean biofilm thickness and density (~31.4% and ~28.3%, respectively) (Figure 3e). At the opposite of crystal violet observations, the association of UTW and PS291 was leading to a higher decrease in biofilm formation with a reduction of the mean thickness of 40.8% and of density of 35.6%. No major rearrangement of the biofilm organization was observed.

The reaction of the RT5 strain of *C. acnes* to UTW PS291 alone or in association was close to that of the RT4 strain (Figure 4). PS was also without significant effect on the thickness and density



**FIGURE 2** Effect of thermal water and rhamnose-rich polysaccharide (PS291®) on the biofilm formation activity of acneic strains of *Cutibacterium acnes* and *Staphylococcus aureus*. The effect of Uriage thermal water (UTW) and PS291® on biofilm formation activity of acneic strains of *C. acnes* RT4 and 5 and of the cutaneous strain of *S. aureus* (MFP03) was measured by the crystal violet technique. Control studies were realized as indicated in Figure 1. All experiments were carried out in at least three replicates (\* $p < 0.05$ )



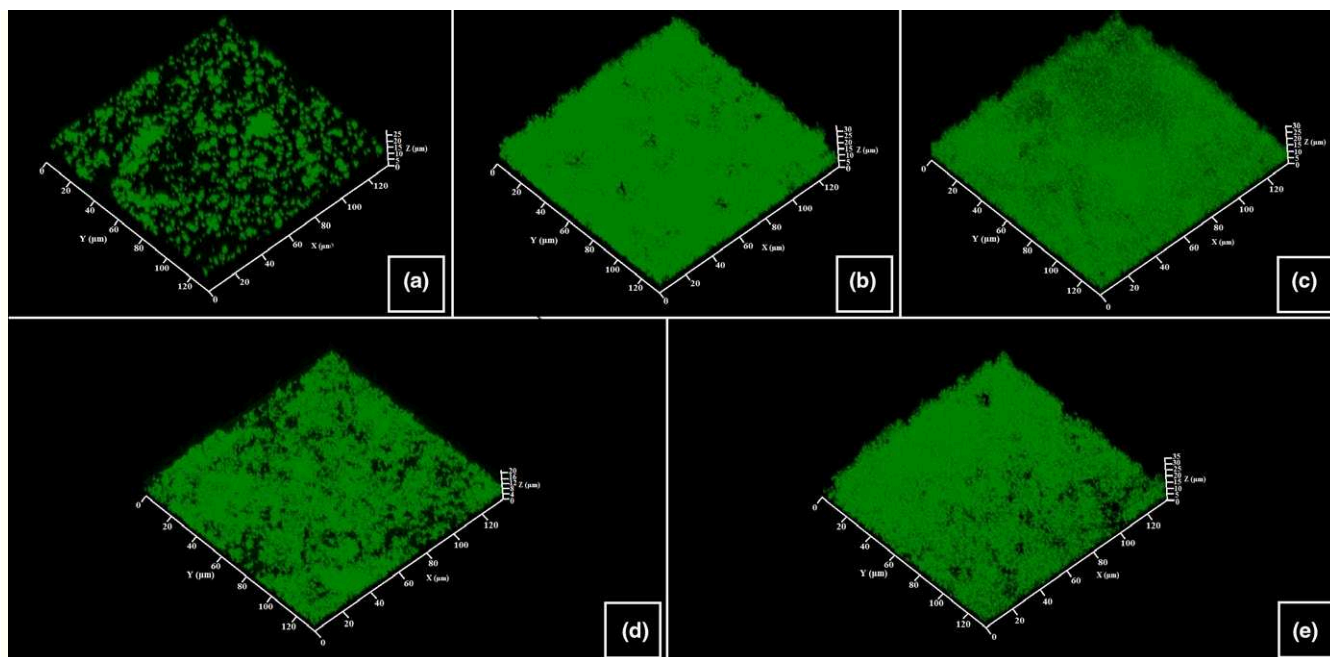
**FIGURE 3** Effect of thermal water and rhamnose-rich polysaccharide (PS291®) on the structure of biofilms formed by the RT4 acneic strain of *Cutibacterium acnes*. Biofilm formation was monitored using Syto 9 green. Horizontal and transverse X/Z views were obtained and submitted to analysis. (a) Control initial cell adhesion after 2 hr. (b) Control biofilm after 72 hr. (c) Biofilm formed in the presence of PS 50%. (d) Biofilm formed in the presence of ETW 50%. (e) Biofilm formed in the presence of PS291 4%. All experiments were carried out in at least three replicates. Control studies were realized as indicated in Figure 1

of the biofilms, a marginal increase was even observed (+4.4% and +4.7%, respectively). The inhibitory effect of UTW and PS291 on the biofilm formation was lower than observed on the RT4 strain with a decrease of the mean thickness of only 14.2% with UTW 50% and only 5.3% with PS291 4%. Even the association of UTW with PS291 was less efficient on the formation of these biofilms (−24.4%). As previously noted the structure of the biofilm was not modified.

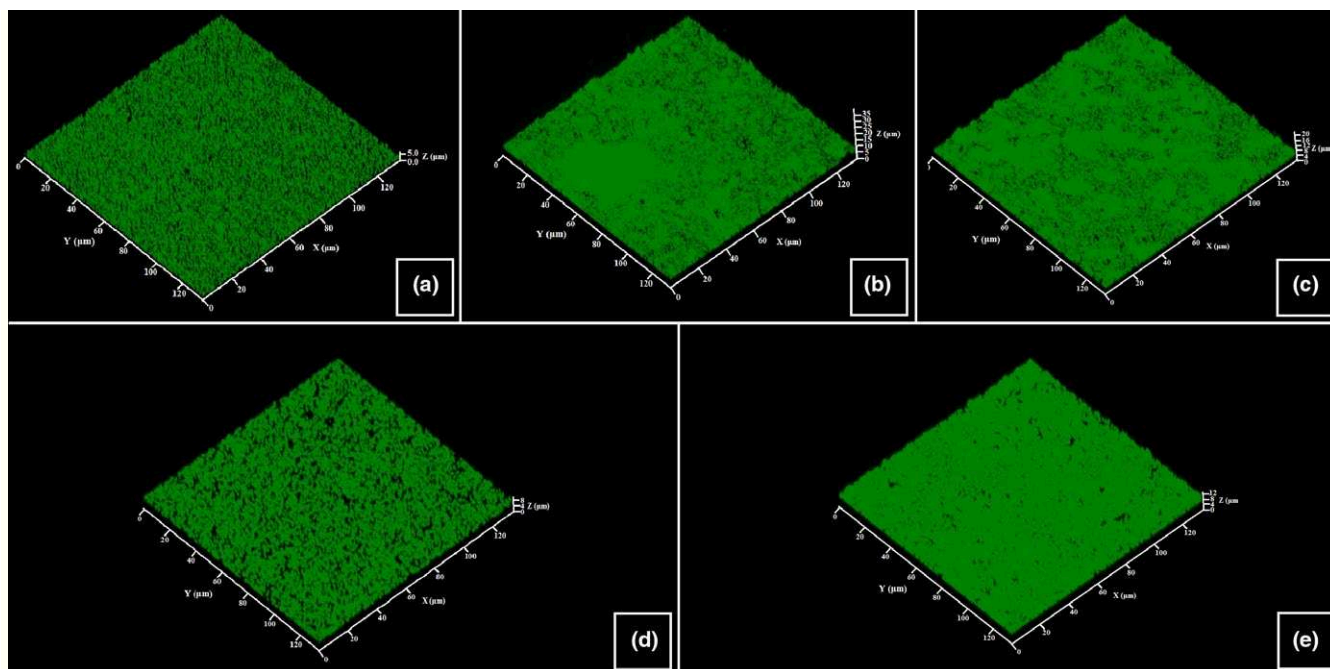
The formation of biofilm by *S. aureus* MFP03 is more rapid than observed with *C. acnes*. Even after 2 hr of initial adhesion an almost complete bacterial mat was observed (Figure 5a). Mature biofilm

was not thick (mean thickness =  $16.7 \pm 1.5 \mu\text{m}$ ) but occasionally showed mushroom-like structure of higher dimension (Figure 5b). As observed with the two *C. acnes* strains, PS 50% did not modify the mean thickness and density of *S. aureus* biofilms. UTW and PS291 reduced *S. aureus* biofilm formation both in terms of mean thickness (−20.3% and −23.7%, respectively) and density (−16.1% and −15.2%, respectively), but this decrease was less important than observed using the *C. acnes* strains. Even the association of UTW with PS291 was less efficient than noted with *C. acnes* with a mean reduction of the biofilm thickness of only 20.9% and no obvious additive effect in comparison of UTW alone.

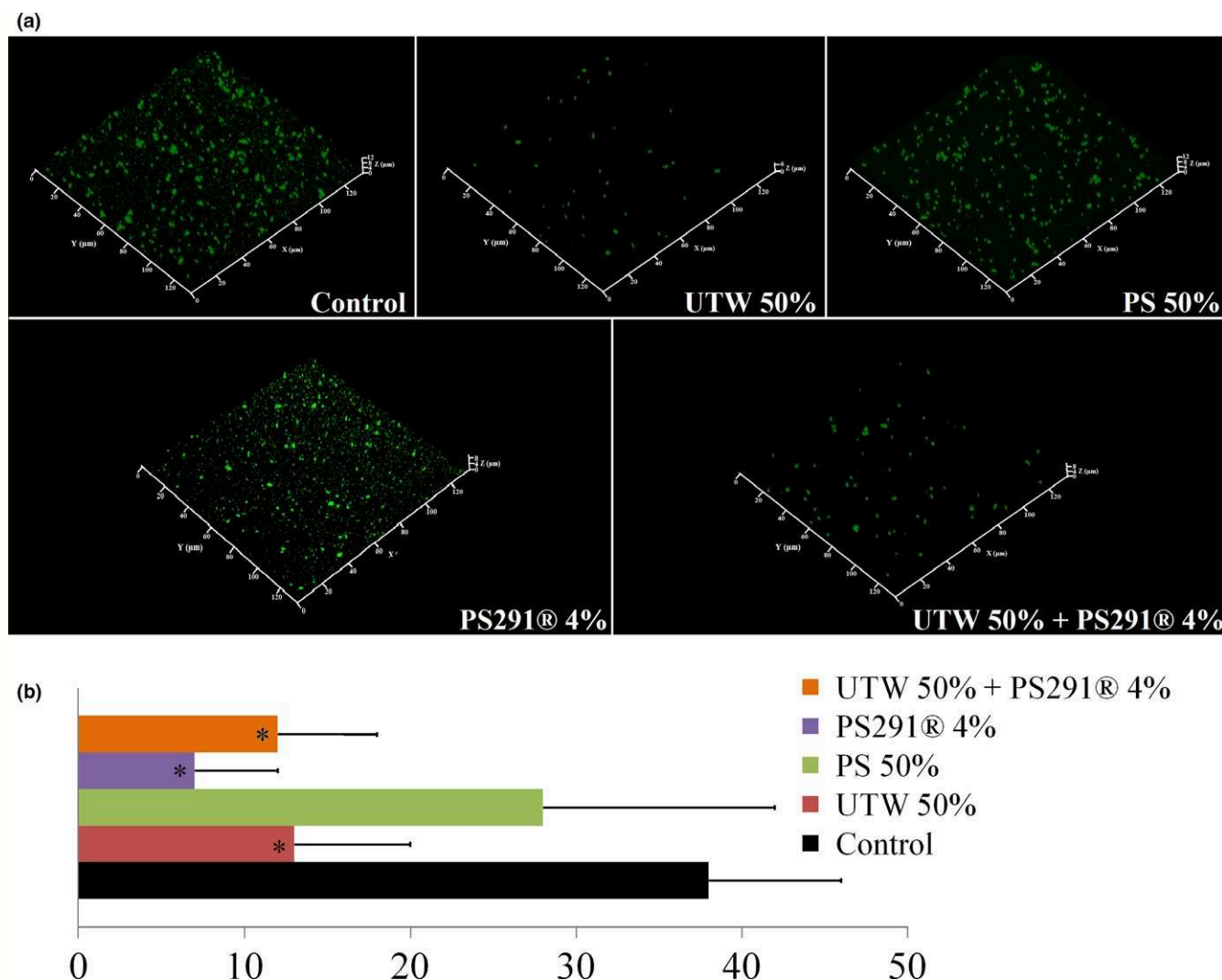




**FIGURE 4** Effect of thermal water and rhamnose-rich polysaccharide (PS291<sup>®</sup>) on the structure of biofilms formed by the RT5 acneic strain of *Cutibacterium acnes*. Biofilm formation was monitored using Syto 9 green. Horizontal and transverse X/Z views were obtained and submitted to analysis. (a) Control initial cell adhesion after 2 hr. (b) Control biofilm after 72 hr. (c) Biofilm formed in the presence of PS 50%. (d) Biofilm formed in the presence of ETW 50%. (e) Biofilm formed in the presence of PS291 4%. All experiments were carried out in at least three replicates. Control studies were realized as indicated in Figure 1



**FIGURE 5** Effect of thermal water and rhamnose-rich polysaccharide (PS291<sup>®</sup>) on the structure of biofilms formed by the human skin strain *Staphylococcus aureus* MFP03. Biofilm formation was monitored using Syto 9 green. Horizontal and transverse X/Z views were obtained and submitted to analysis. (a) Control initial cell adhesion after 2 hr. (b) Control biofilm after 24 hr. (c) Biofilm formed in the presence of PS 50%. (d) Biofilm formed in the presence of UTW 50%. (e) Biofilm formed in the presence of PS291 4%. All experiments were carried out in at least three replicates. Control studies were realized as indicated in Figure 1



**FIGURE 6** Effect of thermal water and rhamnose-rich polysaccharide (PS291®) on *Staphylococcus aureus* MFP03 microcolonies formation in dynamic conditions. In order to investigate the equilibrium between planktonic and adherent bacteria, *S. aureus* MFP03 was incubated in microwells under agitation and microcolonies formation was monitored after 24 hr. (a) Structure of microcolonies formed in the absence of treatment and in the presence of PS 50%, UTW 50%, PS291 4% and UTW 50% + PS291 4%. (b) Mean number of microcolonies formed by *S. aureus*. All experiments were carried out in at least three replicates. Control studies were realized as indicated in Figure 1 (\* $p < 0.05$ )

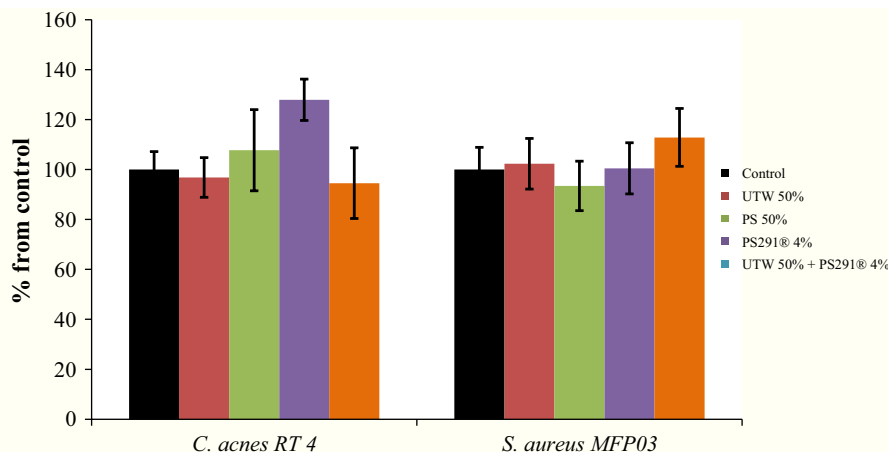
### 3.3 | Effect of ETU and PS241 on *S. aureus* microcolony formation in dynamic conditions

As the initial adhesion step of *S. aureus* was particularly rapid, we used an original approach to investigate a potential effect of UTW and PS291 on the equilibrium between biofilm and planktonic bacteria by growing these cells without preliminary adhesion step and under permanent agitation during 24 hr. This was resulting into the formation of microcolonies on the bottom of the wells (Figure 6a). Cell aggregates with a minimal area of  $2.7 \mu\text{m}^2$  were considered microcolonies and counted. The number of microcolonies in the absence of treatment reached a mean of 38/field ( $14,400 \mu\text{m}^2$ ) (Figure 6b). Addition of UTW 50% resulted in a decrease of to 13/field. In the presence of PS291 4%, the number of microcolonies decreases to 7/field. Conversely, PS 50% only reduced the number of microcolonies to 28/field. The association of UTW with PS291 was not leading to a

additive effect in composition of the effect of the compounds used separately as 12 microcolonies/field were counted in this condition.

### 3.4 | Effect of thermal water and PS291® on the metabolic activity of *C. acnes* and *S. aureus* in biofilms

As the labeling of crystal violet and SYTO9 Green is independent of the viability of bacteria, in order to determine the real amount of metabolically active microorganisms in the biofilm we used an MTT labeling which reveals the oxidative activity of the cells. As shown in Figure 7, PS, UTW and PS291 had limited influence on the viability of *C. acnes* and *S. aureus*. In the presence of PS291, the number of metabolically active cells increased to 119% for *C. acnes* RT4 and to 113% for *S. aureus* MFP03 but this difference was limited although significant. The association of UTW to PS291 did not modify the viability of any of the studied bacteria.



**FIGURE 7** Effect of thermal water and rhamnose-rich polysaccharide (PS291®) on the metabolic activity of an acneic strain of *Cutibacterium acnes* and *Staphylococcus aureus* in biofilms. The Effect of Uriage thermal water and PS291® alone or in association on the metabolic activity of *C. acnes* RT4 and *S. aureus* MFP03 was measured by biofilm staining with 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT). (a) *C. acnes* RT4. (b) *S. aureus* MFP03. All experiments were carried out in at least three replicates. Control studies were realized as indicated in Figure 1

### 3.5 | Effect of thermal water and PS291® on the surface polarity of acneic strains of *C. acnes* and *S. aureus*

Results suggesting that UTW and PS291 could influence the initial binding of bacteria on the surfaces, and this event being dependent of their surface properties, we used the MATS technique to investigate potential surface polarity changes.

Both *C. acnes* strains exhibited high hydrophobicity and affinity to organic all solvents (Figure 8). Exposure to UTW or PS291 alone or in association was not associated to significant changes in the surface properties of *C. acnes* RT4 and RT5 strains. *S. aureus* MFP03 also showed high affinity to chloroform and more relatively to hexadecane and decane and low affinity to ethyl acetate indicating that, as *C. acnes* its surface is hydrophobic. However, the limited affinity to ethyl acetate reveals that the Lewis acid-base properties, and subsequently the surfaces charges of *S. aureus* are not identical to that of *C. acnes*. After exposure of *S. aureus* to UTW 50% and PS291 4%, its affinity to chloroform, hexadecane and decane increased whereas the affinity to ethyl acetate decreased. Chloroform-hexadecane pair having almost identical Van der Waals forces, it appears that UTW and PS291 increased the hydrophobic and electronegative character of the bacterium. This is coherent with the observation of a marked reduction of the affinity of *S. aureus* to ethyl acetate. PS 50% had very different effects on *S. aureus*, particularly in regard of its affinity to chloroform, that was not modified, and hexadecane, that was markedly reduced.

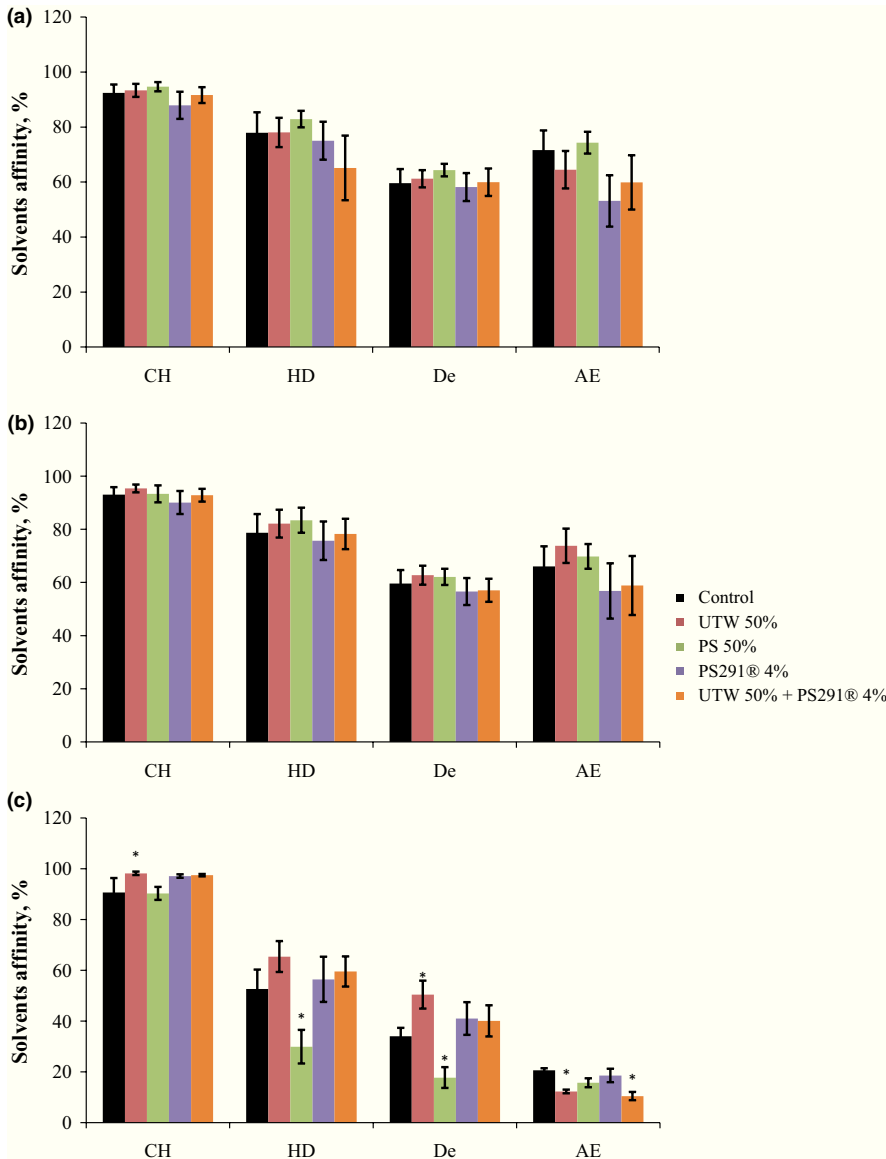
## 4 | DISCUSSION

Effect of active components used, or that will be used, in cosmetics industry have been studied in a number of works. This is an urgent matter in view of a global tendency to develop preservative-free

cosmetic formulae, since preservatives may cause allergy and other skin disorders (Feuilloley, Doléans-Jordheim, & Freney, 2015; Varvaresou et al., 2009). A number of works dealt with the role of *C. acnes* in development of acne simplex and investigation of various compounds suppressing its growth or virulence. Thus, data exist on the inhibitory effect of the extract of cinnamon bark on growth of various skin microorganisms, including *C. acnes* and *S. epidermidis* (Nabavi et al., 2015), on anti-inflammatory effect and inhibition of *C. acnes* by a complex of bakuchiol (a meroterpen isolated from *Psoralea corylifolia*), *Ginkgo biloba* extract, and mannitol (Trompezinski et al., 2016). Magnesium ascorbyl phosphate, a precursor of vitamin C, is known to suppress the processes of lipid peroxide oxidation by *C. acnes* (Lee et al., 2016). The works on *C. acnes* biofilms are however few, and no research has been carried out on the effects of cosmetic preparations on the biofilm growth by acneic forms of *C. acnes*. Similarly, only a few works dealt with the effect of cosmetic components on *S. aureus* biofilms. Extract of unripe *Juglans regia* fruits, which is used in manufacture of hair dyes, was shown to suppress the adhesion capacity and biofilm formation activity of *S. aureus* (Quave, Plano, Pantuso, & Bennett, 2008) and glycerol monolaurate, a natural surfactant used in cosmetics industry, also inhibits the growth of *S. aureus* biofilms (Hess, Henry-Stanley, & Wells, 2014). To the best of our knowledge, this work is the first one presenting a complete study of the effects of cosmetics compounds on the adhesive and biofilm formation activity of acneic strains of *C. acnes* and of a human skin-associated *S. aureus*.

Both acneic strains of *C. acnes* RT4 and RT5 had general similar behavior when they were exposed as planktonic cultures to UTW or PS291. However, whereas PS291 had minor effects on the growth kinetics of these bacteria, UTW was markedly reducing the lag time of the exponential growth phase. Conversely, the final biomass of RT4 was reduced whereas that of RT5 was not modified suggesting that whereas ETU can stimulate the metabolic activity of the





**FIGURE 8** Effect of thermal water and rhamnose-rich polysaccharide on the affinity of acneic strains of *Cutibacterium acnes* and *Staphylococcus aureus* to solvents of different polarities. (a) *C. acnes* RT4. (b) *C. acnes* RT5. (c) *S. aureus* MFP03. chloroform (CH), hexadecane (HD), decane (De) and ethyl acetate (EA). Each value represents the  $M \pm SEM$  of five independent experiments. Control studies were realized as indicated in Figure 1 (\* $p < 0.05$ )

bacteria, a lack of one or several nutrients can limit their final development. UTW was also reducing the final biomass formed by *S. aureus* MFP03 and this effect was identical to that observed using PS, UTW + PS291 or PS + PS291 indicating that the effect of UTW on the final biomass was probably due only to dilution of nutrients in the medium and that the bacteria were unable to use PS291 for their physiological requirements.

It was interesting to see that, as observed with a not characterized strain of *C. acnes* (Enault et al., 2014), UTW and PS291 showed a marked inhibitory effect on the RT4 and RT5 *C. acnes* and *S. aureus* MFP03 biofilm formation activity using the crystal violet technique. PS also decreased the biofilm formation measured by this technique but the effect was lower and the association of ETU with PS291 was more efficient on the biofilm than the association of PS with PS291. Results from confocal scanning microscopy were significantly different as PS was without effect on *C. acnes* RT4 whereas UTW and PS291 inhibited its biofilm formation. *C. acnes* RT5 and *S. aureus* MFP03 reacted to PS, UTW and PS291 as *C. acnes* RT4 strain, except

that the decrease of biofilm formation was more limited. The differences observed between the two techniques should be attributed to the composition of physical properties of the surface on which the biofilms were grown as in the crystal violet technique biofilms were formed on flat-bottomed polystyrene bottom wells whereas in confocal microscopy glass bottom wells were employed and polystyrene is considered as a hydrophobic surface whereas glass is typically hydrophilic (Dagorn et al., 2013). Concerning the *C. acnes* biofilm architecture, both UTW and PS291 affected it in a similar manner. In their presence biofilms formed by both strains were thinner, and the number of projections and structural elements on biofilm surface was reduced (this was actually only visible by direct observation). Thus, the three-dimensional biofilm structure became simplified. It is also interesting to note that in the presence of UTW or PS291 the biofilms were more easily detached in agreement with the postulated anti-adhesive properties of PS291 (Enault et al., 2014) and UTW (Uriage, 2018). The biofilm of *S. aureus* MFP03 formed in the presence of UTW 50% was a monolayer not differing significantly

in thickness from the layer formed during initial adhesion. This effect was observed also for *C. acnes*, although it was less pronounced, possibly because of higher surface hydrophobicity.

In order to go further into the determination of the effects UTW and PS291 on the initial adhesion process of *S. aureus* we used a microcolony formation test in dynamic conditions. This test was not applicable on *C. acnes* RT4 and RT5 strains which required a culture in static and anoxic conditions. UTW and PS291 had a clear inhibitory effect on the initial adhesion step of *S. aureus* whereas PS had a limited effect. Unexpectedly, the association of UTW and PS291 only induced a partial reduction of *S. aureus* initial adhesion, of the same range observed using PS as if in regard of this process the two cosmetic compound had antagonistic effects. That should be explained by the different properties of UTW and PS291, the first one acting on the bacterial surface via its ionic charges and the second by embedding bacteria but therefore inhibiting the ionic interactions.

The absence of toxicity of UTW 50% and PS291 4% on *C. acnes* and *S. aureus* biofilms was verified using the MTT test. Except with PS291 used alone, and in a very limited range, no significant increase of metabolic activity was observed. This almost absence of effect of UTW and PS291 on the metabolic activity of both bacterial species indicates that the tested compounds are not susceptible to provoke dysbiosis in the skin microbiote. This is a requirement of the European Guideline for Cosmetic compounds 1223/2009 (Buzek & Ask, 2009). Nevertheless, MATS tests revealed that UTW and PS291 had a limited but visible effect on the surface polarity of both bacteria leading to a general increase of surface hydrophobicity and electronegative character. These properties are essential in the physical initial adhesion steps of bacteria to surfaces and these results are coherent with confocal microscope observations and microcolonies formation tests. A link between surface polarity and electronegative character and virulence has been previously demonstrated in other bacterial taxonomic groups, such as *Listeria* (Poncin-Epaillard et al., 2013) but the sense, degree of variation, and metabolic response of bacteria in regard of surface polarity is variable between species (Poncin-Epaillard et al., 2013).

Documenting the safety of cosmetic compounds on skin bacteria is nowadays a major industrial challenge with the economic pressure of customers for the development of preservative-free cosmetics (Varvaresou et al., 2009) which therefore could directly affect the skin microbiote. In this regard, UE Regulation No 1223 (Buzek & Ask, 2009) requires that the absence of effect of active cosmetic compounds on the skin microbiote, or at least target microorganism, was controlled to avoid adverse skin reactions. This is particularly important in regard of bacteria such as *C. acnes* and *S. aureus*, which exist both as commensal and pathogenic strains but can also see their biofilm formation activity and virulence changing in response to modifications of the microenvironment (Feuilloley et al., 2015). The metabolic requirements of RT4 and RT5 acneic strains of *C. acnes* in regard of strict anoxic growth conditions are not allowing to study their interactions in binary biofilms with *S. aureus* and therefore the present study was realized on individual biofilms. Nevertheless, this is the first time

that the physiological response of these bacteria to cosmetics was investigated and the present results suggest that UTW and PS291 are safe in regard of *C. acnes* development, although clinical tests should be required to get complete demonstration.

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## AUTHOR CONTRIBUTION

A.G. performed the experiments, analyzed the data, and wrote the draft of the manuscript; V.B. provided technical assistance; L.L., A.I.N., and V.K.P. supervised the work; M.G.J.F. headed funding organization, supervised the work and assisted for manuscript writing. All authors read and approved the final manuscript.

## CONFLICT OF INTEREST

The authors have the following interests. This work was supported by public funds obtained from Evreux Porte de Normandie, Region Normandie and European Union (FEDER). Luc Lefeuvre is employed by the Dermatologic Laboratories Uriage. There are no patents, products in development of marketed products to declare. This does not alter the authors' adherence to all policies on sharing data and materials, as detailed online in the guide for authors.

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## SUPPLEMENTARY DATA FOR THE ARTICLE № 3

Investigation of the effect of cosmetic components on binary biofilms of *S. aureus*MFP03, *S. epidermidis* MFP04 and *C. acnes*

## INTRODUCTION

To determine if there is an effect of cosmetic compounds UTW™ and PS291® on binary biofilms of skin microbiota, we also conducted a series of experiments with binary systems *S. aureus* MFP03 + *S. epidermidis* MFP04 and *S. aureus* MFP03 + *C. acnes* RT5.

## MATERIALS AND METHODS

In the case of binary systems of staphylococci, the experiments were carried out in glass test tubes. Briefly: 6 ml of TSB medium with 0.25% glucose with 50% UTW additions (50% PS as control) or 4% PS291® were added to the tubes, 100 µl of cell suspension of each microorganism was added. Simultaneously, plating of mono-species cultures was carried out. Biofilms were grown for 24 hours on a shaker at 37°C. At the end of the incubation period, one sample from each variant was used to determine the number of CFUs, and the second one was used to measure the metabolic activity of the cells.

In the case of *S. aureus* MFP03 and *C. acnes* RT5 binary systems, biofilms were grown in 12-well polystyrene plates (Thermo Scientific) on glass fiber filters in a liquid RCM medium. Biofilms were grown for 72 hours at 33°C under anaerobic conditions (GasPak®). At the end of the incubation period, the manipulations described above were performed.

## RESULTS

Studies of potential effect of cosmetic compounds on binary biofilms have been conducted with use of liquid medium in small volumes. Also, to minimize possible errors associated with the transfer of carriers with biofilms from old media to new ones (as in experiments with preformed biofilms), only biofilms formed simultaneously by both microorganisms were examined.

In the binary planktonic cultures of *S. aureus* MFP03 and *S. epidermidis* MFP04 in this system, the total amount of CFU of both microorganisms in the control was  $5.2 \times 10^{10}$  cells, of which *S. aureus* MFP03 is  $3.9 \times 10^{10}$ , and *S. epidermidis* MFP04 is  $1.3 \times 10^{10}$ : the difference in quantity CFU was 2.6 times (Figure 1-A). Adding 50% of the FR to the medium decreased the amount of CFU due to dilution and lack of nutrients: the total number of CFUs was  $2.18 \times 10^{10}$ , *S. aureus* MFP03 -  $8.9 \times 10^9$ , *S. epidermidis* MFP04 -  $1.29 \times 10^{10}$ . The difference in the amount of CFU of *S. aureus* and *S. epidermidis* was 0.69. However, with the addition of UTW 50%, the amount of CFU of both microorganisms increased in comparison with the control: the total number of CFUs was  $1.3 \times 10^{11}$ , of which *S. aureus* MFP03 was  $6.95 \times 10^{10}$  CFU, and *S. epidermidis* MFP04 was  $6.05 \times 10^{10}$ , the difference in the number of CFU microorganisms was 1.15 times.

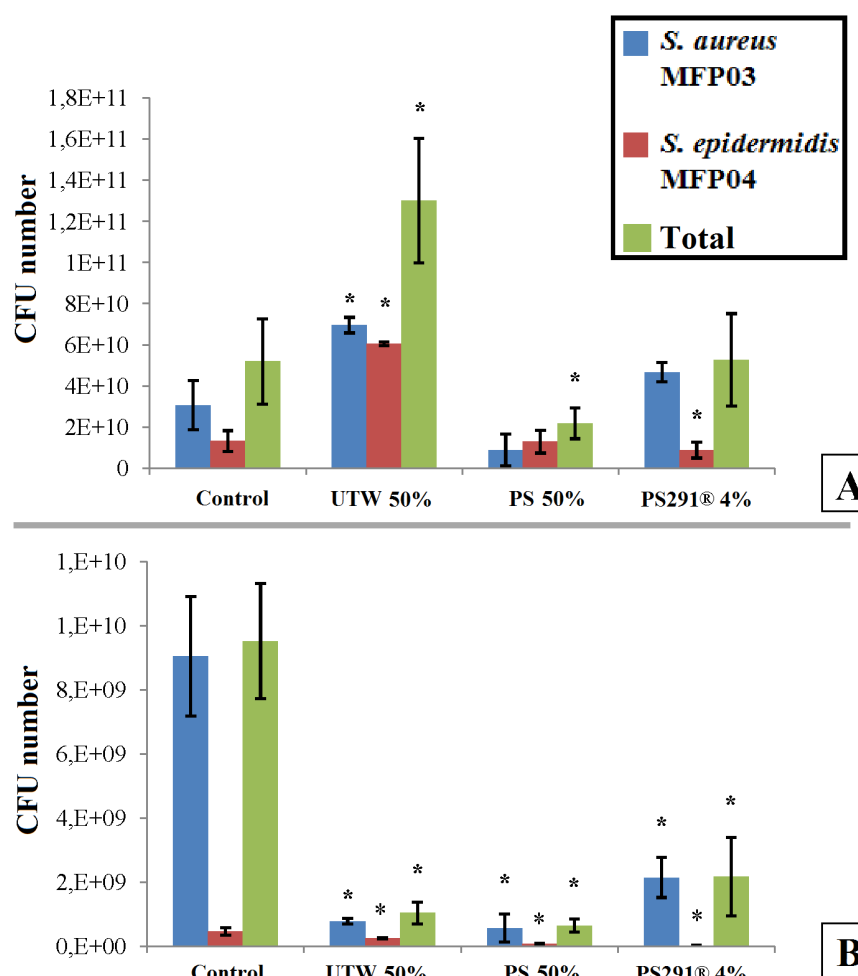


Fig. 1. The amount of CFU of *S. aureus* MFP03 and *S. epidermidis* MFP04 in binary planktonic cultures and biofilms with the addition of UTW 50%, PS 50%, PS291® 4%. A - planktonic cultures. B - biofilms. \* -  $p = 0.05$ .

Thus, the UTW, despite dilution of the medium, increased the amount of CFU in planktonic cultures. At the same time, the effect of medium dilution is expressed in a larger increase in CFU of *S. epidermidis* MFP04 relative to *S. aureus*. This is also confirmed by the results of CFU counting of samples with PS291®. Since there was no dilution of the medium, the difference in the number of CFU of microorganisms was 5.2 times (the number of CFU of *S. aureus* MFP03  $4.67 \times 10^{10}$ , the number of CFU of *S. epidermidis* MFP04 -  $8.78 \times 10^9$ , total -  $5.5 \times 10^{10}$ ). In this case, the amount of CFU of *S. aureus* in the presence of PS291® increased with respect to the control. If we compare the amount of CFU in mono-species and binary plankton cultures, then *S. aureus* MFP03, then a significant difference in the number of CFU between mono-species and binary plankton cultures was observed only with the addition of 50% PS, when it decreased in binary culture 3-fold compared to mono-species. In general, both UTW and PS291 increased the number of CFUs of *S.*

*aureus* MFP03 in comparison with the control in both mono-species and binary plankton cultures. The addition of a 50% PS also increased the number of CFUs in mono-species cultures compared to control, but decreased in binary ones. *S. epidermidis* MFP04 grew significantly worse in binary cultures than in mono-species (Figure 4): the difference in the number of CFU of *S. epidermidis* MFP04 between mono-species and binary plankton cultures was 16 times. The addition of 50% UTW increased the number of CFUs both in mono-species (about 2.5 times) and in binary cultures (about 4.5 times), and the number of CFUs in mono-species cultures was only 9 times higher than in binary cultures. The smallest difference (only 4 times) between the number of CFU of *S. epidermidis* MFP04 in mono-species and binary biofilms was with the addition of 50% PS: in binary cultures, the amount of CFU remained at the control level, while in mono-species it declined (which confirms the hypothesis of the effect of medium dilution on ratio of microorganisms in binary cultures). The addition of PS291® did not significantly affect the balance of microorganisms in binary cultures compared to the control, nor did it significantly affect the amount of CFU in mono-species plankton cultures.

In biofilms (Figures 1-B, 4), the number of CFUs of both microorganisms was significantly smaller: in the control, the total number of CFUs averaged  $9.52 \times 10^9$  (which is almost an order of magnitude lower than in plankton culture), and This amount was  $9.05 \times 10^9$  CFU *S. aureus* MFP03 (20-fold advantage over *S. epidermidis* MFP04). The addition of PS, UTW, and PS291® greatly reduced the amount of CFU in binary biofilms, primarily by reducing the amount of CFU of *S. aureus*, but also the amount of CFU of *S. epidermidis* also decreased compared to the control. With the addition of UTW, the number of CFUs of *S. aureus* MFP03 and *S. epidermidis* MFP04 was  $7.9 \times 10^8$  and  $2.5 \times 10^8$ , respectively (difference 3.16 times), and with the addition of PS -  $5.7 \times 10^8$  and  $8.3 \times 10^7$ , respectively (difference 6.9 times). Addition of PS291® increased the difference in the amount of CFU between *S. aureus* and *S. epidermidis* up to 77 times ( $2.15 \times 10^9$  vs.  $2.8 \times 10^7$  CFU, respectively). Thus, UTW increased the competitive properties of *S. epidermidis* MFP04 in biofilms with *S. aureus* MFP03, and PS291® - weakened.

When comparing the number of CFU strains between mono-species and binary biofilms, the following pattern was observed: when all the tested components of cosmetics and PS were added to *S. aureus* MFP03, the amount of CFU in mono-species and binary biofilms decreased (Figure 4-B), whereas in *S. epidermidis* MFP04 in monofilament biofilms when adding TU, the amount of CFU increased, with addition of PS - decreased, with the addition of PS291 ® - did not change (Figure 4-D), which indicates the opposite effects of these compounds on the growth of biofilms of two closely related microbes. This confirms the hypothesis of an increase in the competitive properties of *S. epidermidis* MFP04 with respect to *S. aureus* MFP03 in the presence of UTW. PS291,



apparently, does not have such an effect. This is also confirmed by the data on the staining of biofilms with MTT (data not shown): when the test compounds were added, the OD of MTT extracts from biofilms decreased.

### 3.6.2. Investigation of the effect of cosmetic components on binary biofilms *C. acnes* RT5 and *S. aureus* MFP03

In studies, the concentrations of UTW (and, correspondingly, PS) were 30% by volume, since in this concentration they had practically the same effect as at 50% (data not shown). In the course of the experiments, it was found that the number of CFU of *S. aureus* MFP03 in mono-species biofilms was significantly reduced by the addition of UTW and PS, which apparently is due to dilution of the medium ( $1.76 \times 10^9$  in the control of  $1.2 \times 10^9$  and  $1.1 \times 10^9$  with addition of UTW and PS, figure 2). PS291® also reduced the amount of CFU in mono-species biofilms ( $1.6 \times 10^9$ ), however, the effect was not as strong as in the case of TSS.

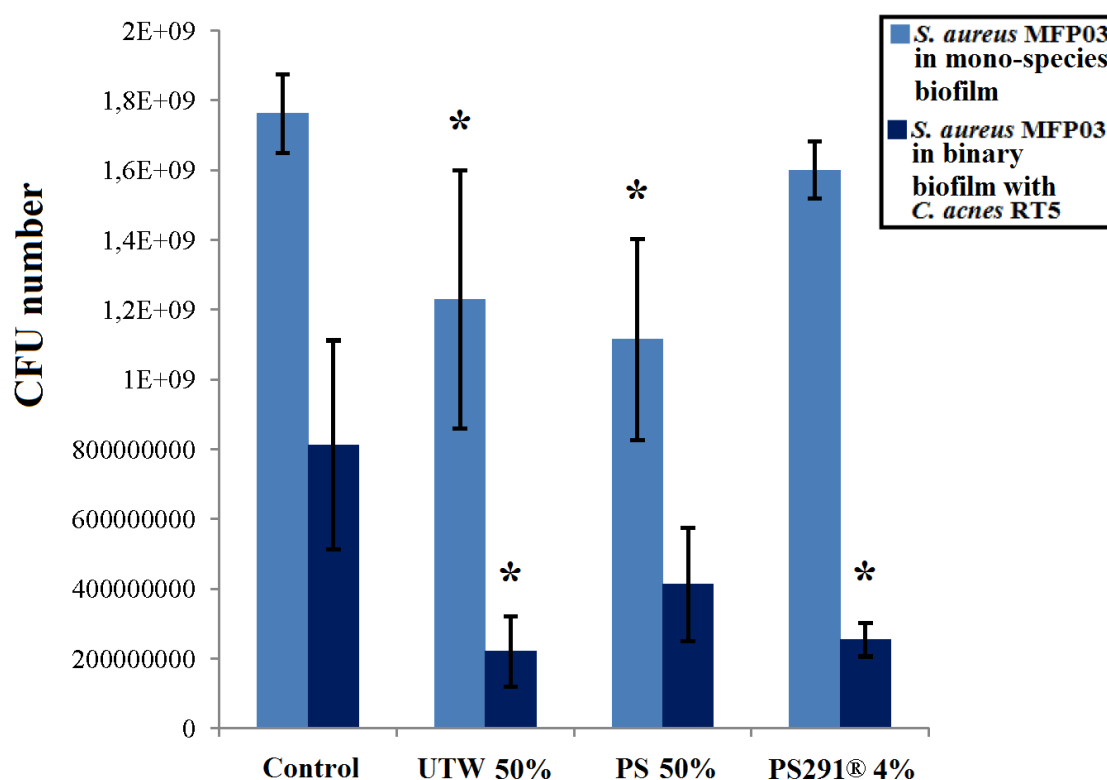


Fig. 2. The amount of CFU of *S. aureus* MFP03 in mono-species and binary biofilms with *C. acnes* RT5 with addition of 30% UTW, 30% PS, 4% PS291®. \* -  $p = 0.05$ .

Biofilms were grown at  $33^\circ \text{C}$  - the natural temperature of some skin areas. In binary biofilms in the control, the amount of CFU of *S. aureus* MFP03 was 2.15 times lower than in mono-species ( $8.1 \times 10^8$ ). The addition of 30% UTW increased the difference in the amount of CFU of *S. aureus* MFP03 between mono-species and binary biofilms to 5.6 times ( $2.21 \times 10^8$  CFU *S. aureus* MFP03 in

binary biofilm with addition of UTW). PS increased the difference in the number of CFUs of *S. aureus* MFP03 between monovisual and binary biofilms lisch up to 2.7 times. The greatest increase in the difference in the number of CFUs was the addition of PS291®: in binary biofilms, the number of CFU of *S. aureus* MFP03 decreased 6.3 times (up to  $2.54 \times 10^8$  compared to mono-viruses). The results indicate that the cosmetic ingredients weakened the competitive properties of *S. aureus* MFP03 in the binary system with *C. acnes* RT5. These findings confirmed the results of the staining of MTT biofilms (Figure 3).

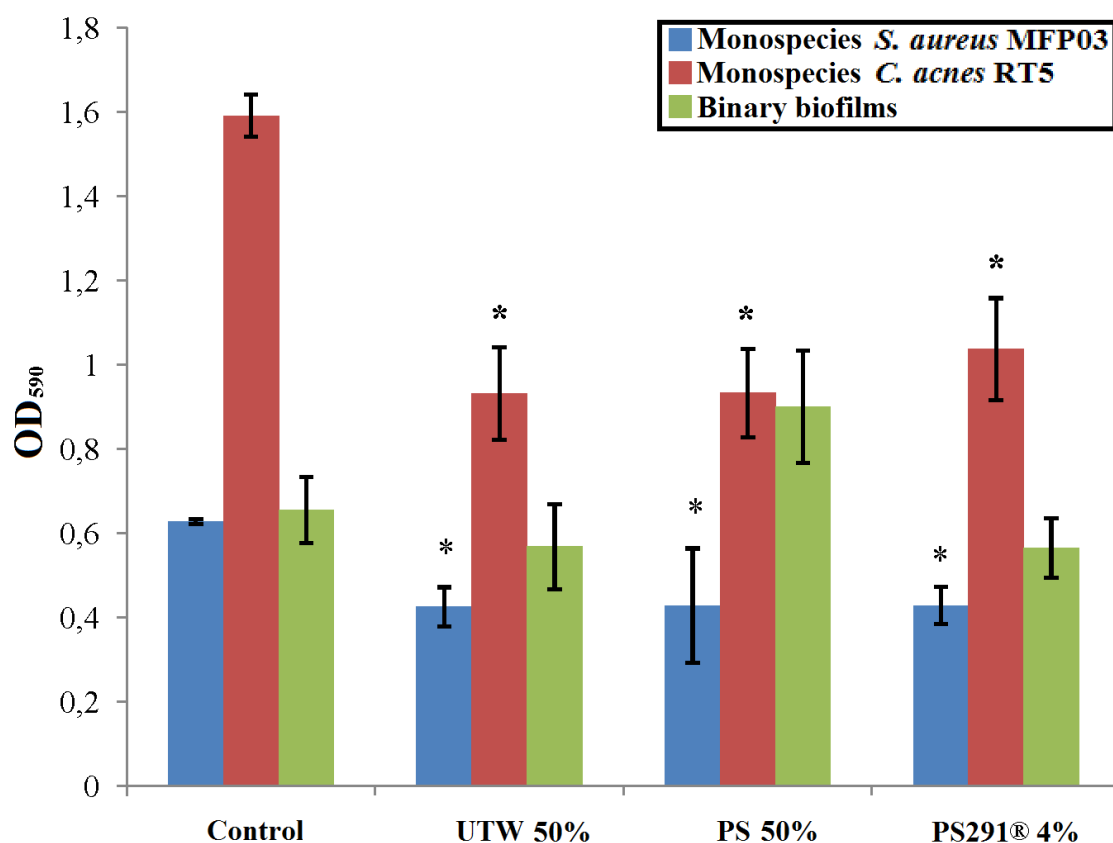


Fig. 3. MTT staining of mono-species and binary biofilms of *C. acnes* RT5 and *S. aureus* MFP03, grown in the presence of components of cosmetics. \* -  $p = 0.05$ .

Both components of cosmetics and PS reduced the metabolic activity (equivalent to the OD of the formazan extract) of the mono-species biofilms of both microorganisms by reducing the number of CFU in biofilms. In control, the metabolic activity of cells in binary biofilms was almost at the level of *S. aureus* MFP03 mono-species biofilms, which indicates the dominance of *S. aureus*. Because UTW and PS291® reduced the competitive advantage of *S. aureus* in binary biofilms, the metabolic activity in binary biofilms in the presence of UTW and PS291® was also much lower than the sum of the activities of mono-species biofilms, but higher than the metabolic activity of *S. aureus* monofilament biofilms: probably, the amount of *C. acnes* increased. With the addition of the



PS, the competitive properties of *S. aureus* did not decrease, however, due to the fact that *S. aureus* grew worse than in control, it appears that *C. acnes* gained an advantage here. Because of these two circumstances, the metabolic activity of binary biofilms was significantly higher than in control.

Thus, summing up the subtotal, it can be said that UTW and PS291® reduce not only the amount of biomass of biofilms, but also weaken the competitive properties of *S. aureus* MFP03, which may be an important property of these compounds as potential regulators of cutaneous microbiota.

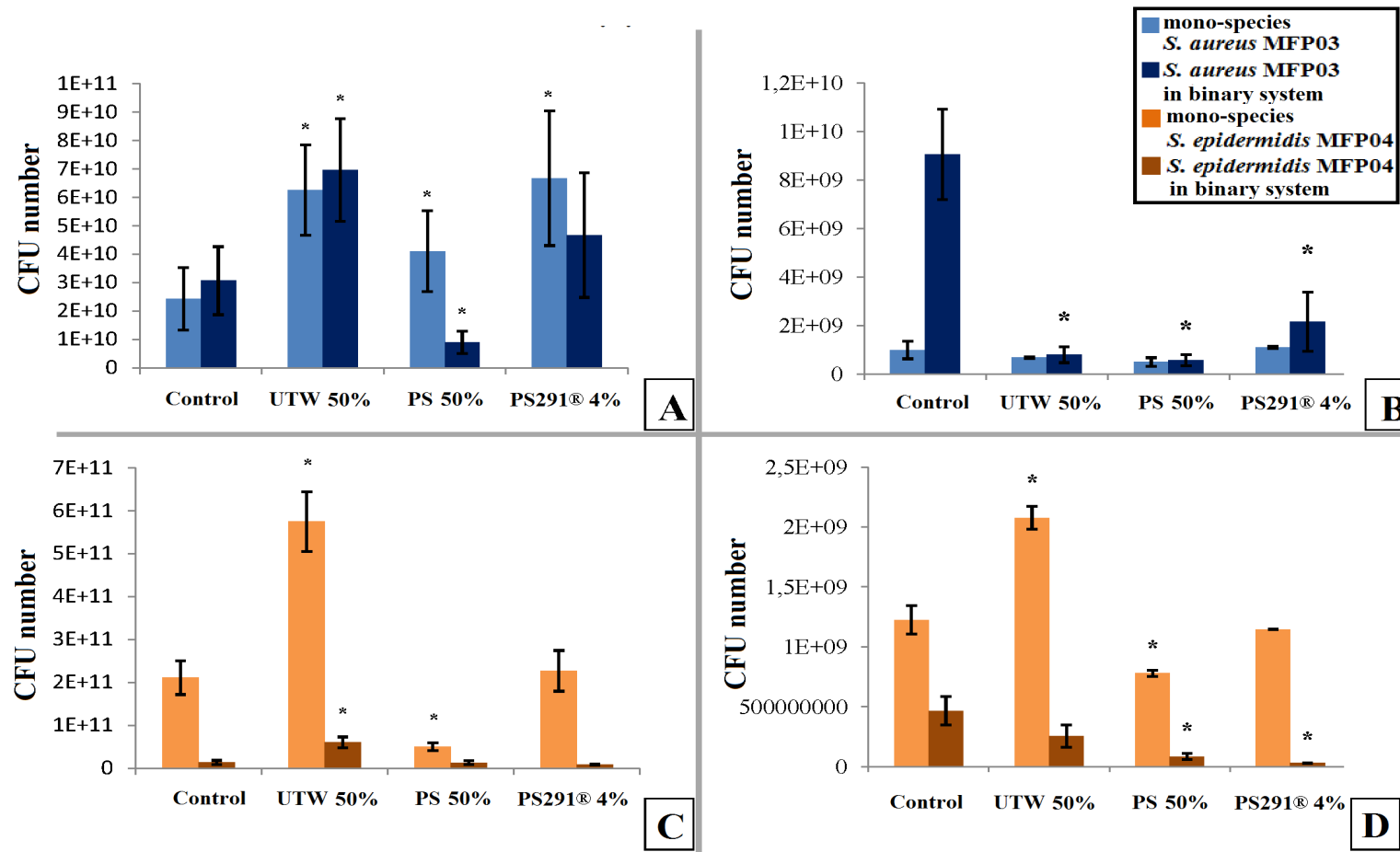


Fig. 4. Comparison of the number of CFUs of *S. aureus* MFP03 and *S. epidermidis* MFP04 in mono-species and binary planktonic cultures and biofilms in the presence of cosmetic components. A - the number of CFU of *S. aureus* MFP03 in planktonic cultures; B - number of CFU of *S. aureus* MFP03 in biofilms; C - number of CFU of *S. epidermidis* MFP04 in planktonic cultures; D - number of CFU *S. epidermidis* MFP04 in biofilms.

## CONCLUSION

We investigated the effect of UTW and PS291® on the growth of binary biofilms of *S. aureus* MFP03 and *C. acnes* RT5, as well as *S. aureus* MFP03 and *S. epidermidis* MFP04. The addition of UTW to the culture medium increased the competitive properties of *S. epidermidis* MFP04 in binary biofilms with *S. aureus* MFP03 compared to the control, and also increased the competitive properties of *C. acnes* RT5 in binary biofilms with *S. aureus* MFP03. PS291® had a different effect: in *S. aureus* MFP03 and *S. epidermidis* MFP04 binary biofilms, it suppressed the growth of *S. epidermidis* compared to control, and in *C. acnes* RT5 + *S. aureus* MFP03 binary biofilms it increased the competitive properties of *C. acnes* RT5, partially worsening the growth of *S. aureus* MFP03. This can be one of the reasons for the beneficial effect of TSS and PS291® on the skin, as they disrupt the growth of biofilms considered a more aggressive pathogen of *S. aureus*. It can be noted that in the case of *C. acnes* RT5 and *S. aureus* MFP03 binary biofilms, the effect of both cosmetic products differs from that in monofluid biofilms, where both compounds suppress the growth of biofilms of both microorganisms.

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EXPERIMENTAL ARTICLES

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## Regulation of Formation of Monospecies and Binary Biofilms by Human Skin Microbiota Components, *Staphylococcus epidermidis* and *Staphylococcus aureus*, by Human Natriuretic Peptides

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**Abstract**—Human natriuretic peptides (NUPs) types A and C were shown have different effects on the growth of monospecies and binary biofilms of human skin commensal microorganisms *Staphylococcus epidermidis* and *Staphylococcus aureus*. The effect of natriuretic peptides depended on cultivation conditions: at 37°C growth of monospecies biofilms formed by *S. epidermidis* and *S. aureus* was stimulated and inhibited, respectively, resulting in changed values of the biofilm average thickness and biomass in presence of NUPs. At 33°C effects of NUPs reversed: growth of *S. epidermidis* monospecies biofilms was inhibited. In binary biofilms at 37°C, NUPs were able to increase the competitiveness of *S. epidermidis* against *S. aureus*. NUPs affected predominantly biofilms and, to a lesser degree, planktonic cultures. This phenomenon makes it possible to consider NUPs as the molecules able to regulate the interactions between the human organism and skin microbiota.

**Keywords:** natriuretic peptides, biofilms, binary biofilms, *Staphylococcus aureus*, *Staphylococcus epidermidis*, regulation of biofilm formation

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Numerous papers published worldwide deal with human skin microbiota and the implications of its interaction with the host. The skin microbiota is a complex community of microorganisms intensely interacting with one another and with host tissues (Chiller et al., 2009; Belkaid et al., 2014; Prescott et al., 2017). A widespread commensal human skin inhabitant is *Staphylococcus epidermidis* that prefers moist skin areas. It inhabits both the skin surface (Grise et al., 2009) and the cavities of cutaneous glands and hair follicles (Grise et al., 2009; Laureano et al., 2014). *S. epidermidis* is an opportunistic pathogen. It may be useful or at least do no harm to the human organism under certain conditions (Christensen and Bruggemann, 2014); otherwise, it can cause an infection (Sandvik et al., 2013; Sabate Bresco et al., 2017). A closely related species, *Staphylococcus aureus*, is generally believed to be a more dangerous and aggressive microorganism that is involved in skin diseases (Brown et al., 2013). Nonetheless, there is somewhat ambiguous evidence that *S. aureus* may be a component of the normal skin microbiota of a part of the human population (Feuerstein et al., 2017). The two closely related species can intensely interact. They

form a part of multispecies communities such as biofilms (Vandecastelaere et al., 2017). Biofilms, microbial communities embedded in the extracellular matrix (Nikolaev and Plakunov, 2007), can cause a number of human diseases; as is the case with *S. aureus* (Matard et al., 2013) and *S. epidermidis* (Otto, 2012) biofilms. However, there is evidence that *S. epidermidis* can exert a protective effect by competing with *S. aureus* (Iwase et al., 2010; Sugimoto et al., 2013) and by modifying its metabolic processes in biofilms (Vandecastelaere et al., 2017).

One of the strategies of treating skin microbiota-induced diseases is based upon using antimicrobial substances. Due to the present-day depletion of antibiotic resources, the quest for new compounds that influence multispecies biofilms in the human organism and make it possible to regulate their growth is a promising approach. Of considerable interest are not only well-known non-antibiotic antimicrobials produced, e.g., by the human organism, such as defensins, but also the substances that have no direct antimicrobial effect and, therefore, more seldom cause emergence of resistant microbial forms. *Escherichia coli* and *Pseudomonas aeruginosa* possess receptors to

epinephrine, norepinephrine, and other human neurochemicals that regulate a wide variety of processes in bacteria including growth, toxin synthesis, pili formation, etc. (Hughes and Sperandio, 2008). The endocrine system-produced hormones are not the only compounds with a significant effect on human microbiota. Substance P, one of the main skin neuropeptides that is synthesized in skin neuron terminals (Severini et al., 2002), plays an important role in regulating a number of processes in such members of skin microbiota as *S. aureus* and *S. epidermidis*. In the presence of substance P, the synthesis of staphylococcal enterotoxin C2 is increased in *S. aureus* and biofilm growth is enhanced in *S. epidermidis* (N'Diaye et al., 2017). Elongation factor EF-Tu is the substance P receptor in staphylococci (N'Diaye et al., 2017). Moreover, substance P, neuropeptide Y, and a number of other neuropeptides exhibit antibacterial activity against *E. coli*, *Enterococcus faecalis*, and *Lactobacillus acidophilus* (El Karim et al., 2008).

Of particular interest are natriuretic peptides (NUPs). There are three NUP types: the A type (atrial natriuretic peptide, ANP), the B type (brain natriuretic peptide, BNP), and the C type (cardiac natriuretic peptide, CNP) (Potter et al., 2009). It was established earlier that CNP affects toxin synthesis in *P. aeruginosa* (Blier et al., 2011). CNP and BNP to some extent suppress pyocyanin synthesis (Blier et al., 2011) and modify lipopolysaccharide synthesis (Veron et al., 2007) in *P. aeruginosa*. They also increase *Pseudomonas fluorescens* cytotoxicity, which apparently results from the involvement of NUPs in the synthesis of cyclic nucleotides (Veron et al., 2007, 2008). *P. aeruginosa* was shown to contain a CNP receptor, a C type amidase (Rosay et al., 2015). NUPs are synthesized by various human cells. They circulate in the bloodstream and, therefore, produce an effect on the skin microbiota in areas with a dense blood capillary network. Hence, the two skin glands-inhabiting microorganisms are constantly exposed to the influence of NUPs. This may affect their growth and biofilm formation and therefore, the state of the skin in general.

The goal of the present work was to investigate the impact of NUPs on the growth of the planktonic cultures and monospecies and binary biofilms of *S. epidermidis* and *S. aureus* under cultivation conditions that simulate those of the skin in various physiological states.

## MATERIALS AND METHODS

### Tested strains and their cultivation and preparation.

The *S. epidermidis* MFP04 and *S. aureus* MFP03 strains were isolated from the skin of healthy volunteers. The strains were characterized using proteomics (MALDI-TOF) and the 16S rRNA gene sequencing (Hillion et al., 2013). Long-term storage of the cultures was carried out at  $-80^{\circ}\text{C}$ . For the experiments,

thawed cultures were plated on petri dishes with lysogenic broth (LB) agar and incubated at  $37^{\circ}\text{C}$ . The resulting separate colonies were used for obtaining 24-h cultures in liquid LB medium at  $37^{\circ}\text{C}$ . The inoculum was prepared by washing the cells twice with sodium chloride physiological solution (PS), which was used to bring the cell suspension to a final optical density of 1 at a wavelength of 580 nm and a path length of 1 cm.

**Natriuretic peptides.** The following two natriuretic peptides (NUPs) were tested: the human A type peptide, ANP (Alfa Aesar, United States) with a molecular weight of 3080.47 Da and the human C type peptide, CNP (PolyPeptide, Sweden) with a molecular weight of 2196.1 Da. Initially, the stock peptide solutions were prepared: sterile MilliQ water (MQ) was applied to the manufacturer's vial with the peptide. The molar concentration of the stock ANP and CNP solutions was  $1.623 \times 10^{-4}$  and  $4.554 \times 10^{-4}$  M, respectively. The stock solutions were divided into aliquots in 500- $\mu\text{L}$  Eppendorf tubes and stored at  $-20^{\circ}\text{C}$ . In compliance with the literature data on the physiological peptide concentration in the human organism (Edwards et al., 1988; Stingo et al., 1992) and the NUP concentrations used in the studies with eukaryotic cells (Klinger et al., 2013), we tested the following three concentrations of each peptide:  $1 \times 10^{-6}$ ,  $1 \times 10^{-7}$ , and  $1 \times 10^{-8}$  M. A mixture containing  $10^{-6}$  M of each peptide was also used. Immediately before inoculating the culture into the nutrient medium, the stock peptide solution or the solution obtained by diluting it 10 or 100 times (yielding the concentrations of  $1 \times 10^{-7}$  and  $1 \times 10^{-8}$  M, respectively) was added.

**Analysis of culture growth dynamics in the presence of NUPs.** The growth dynamics of monospecies cultures was monitored in a Bioscreen C system (Finland) in Honeycomb 100-well immunological plates (Grow Curves, United States). NUP solutions were applied to the wells that were thereupon supplemented with a nutrient medium, tryptone–soybean broth (TSB) with 0.25% glucose (wt/wt). The final liquid volume in a well was 200  $\mu\text{L}$ . The inoculum was prepared beforehand and dispensed to each well (15–20  $\mu\text{L}$ ), so as to obtain a final mixture optical density of 0.08–0.1 at a wavelength of 580 nm. The cultures were grown for 24 h under aerobic conditions, and the optical density was measured at 15-min intervals. To elucidate the impact of cultivation conditions (for modeling various physiological skin states), the cultures were grown at the following temperatures:  $37^{\circ}\text{C}$  (the routine laboratory temperature that equals the skin temperature during inflammation) and  $33^{\circ}\text{C}$  (the average facial skin temperature) (Ariyaratnam and Rood, 1990). Some of the wells remained uninoculated as the negative control, and those inoculated without adding the peptides served as the positive control. The cell doubling time, the growth constant in the

presence of the peptides, the lag phase duration, the cell death constant, and the maximum OD value as a cell yield criterion were determined.

**Monitoring biofilm formation by crystal violet staining.** The effect of NUPs on monospecies biofilm formation was assessed using a modified classical technique (O'Toole, 2011). After the end of the incubation time of the plates with cultures in the Biocreen system, the planktonic culture was removed and the biofilms on the plate walls and bottom were three times washed with PS, dried at 60°C for 15 min, and fixed with 96% ethanol for 20 min at room temperature. Alcohol was removed after fixation, and the biofilms were dried at 60°C and stained with 1% solution of crystal violet (CV; it stains both bacterial cells and the biofilm matrix) for 20 min. After staining, the CV solution was discarded and the wells were washed six times with distilled water. Thereupon, the biofilm-bound dye was extracted with 200  $\mu$ L of 96% ethanol for 60 min. Optical density of the extract was measured at  $\lambda = 600$  nm in the Bioscreen system. The result was presented in the form of the per cent ratio between the OD of the NUP-containing sample and that of the peptide-untreated control sample.

**Confocal laser scanning microscopy (CLSM).** NUP-induced changes in the structure of monospecies biofilms were examined under an LSM 710 Confocal laser scanning microscope (Zeiss, Germany). The biofilms were formed in Sensoplate 24-well plates with a flat bottom (Geiner bio-one, Germany), using a modified method of Coenye et al. (2007). Plates with a glass bottom are widely used in CLSM studies because their glass has no intrinsic fluorescence within the working wavelength range of CLSM lasers, in contrast to plastic (Piruska et al., 2005). Brief experimental protocol was as follows: upon preparation, the inoculum (300  $\mu$ L) was dispensed into wells and incubated for 2 h at room temperature (preliminary cell adhesion). Thereupon, the liquid was removed, the wells were washed two times with sterile PS to remove unattached cells, and one well was fixed with 96% ethanol (the preliminary adhesion control sample). All wells except the fixed one were supplemented with glucose-containing TSB medium with or without added peptides (the control sample). The plates were incubated at 37 or 33°C for 24 h under stationary conditions. Thereupon, the liquid was discarded and the wells were washed twice with PS to remove unattached cells. The samples were stained with the SYTO 9 Green nonspecific fluorescent DNA dye (Molecular probes (Thermo Fisher Scientific)): the manufacturer-produced dye solution in DMSO was 1000 times diluted, and 300  $\mu$ L of the solution obtained was applied to the wells, including the fixed well. The staining was performed for 20 min at room temperature in the dark, whereupon the liquid was discarded and the wells were washed two times with PS. Prolong Diamond Antifade Mountant fixating liquid (1–2 drops, Molecular probes (Thermo Fisher Scien-

tific)) was added, and the system was incubated overnight at 4°C. The samples were analyzed at a laser wavelength of 488 nm with a 63 $\times$ /1.4 oil immersion lens. The microscopic studies were conducted and the sample data files were obtained using the Zen 2009 software package (Carl Zeiss, Germany). The data obtained were processed using the ImageJ package in the Comstat2 plug-in software (based on pre-designed computational algorithms). It was used for determining the following two parameters: biofilm average thickness ( $\mu$ m) and average biomass distribution per area unit ( $\mu$ m<sup>3</sup>/ $\mu$ m<sup>2</sup>). The results were expressed as per cent values relative to the positive control sample (without additions), which was considered to be 100%. The parameters were calculated automatically: the plug-in software used the digital data of its own image files that were directly obtained using Zen2009 SW.

**Investigation of the NUP effect on the ratio of two bacterial species and on the cellular metabolic activity in binary cultures and biofilms.** TSB with glucose (6 mL) and two pre-autoclaved (1 atm) round Whatman GF/F glass-fiber filters with a diameter of 21 mm (Sigma) were placed in sterile screw-capped glass test tubes. The filters were arranged along the test tube wall in such a way that they were at the same level, completely covered with the medium, and out of contact with one another. Thereupon, the test tubes were supplemented with required peptide amounts; the control sample lacked the additions. After the preparation procedure, the inoculum was diluted with sterile PS to OD<sub>580</sub> 0.5; 100  $\mu$ L of the inoculum were applied to test tubes. Comparative studies were concomitantly conducted with binary and monospecies cultures and biofilms. An aliquot of the inoculum was plated on petri dishes with TSB agar to determine the colony-forming unit (CFU) number. The CFU value of the inoculum was subsequently used to calculate the CFU increase in biofilms and suspension cultures. After 24 h of incubation at 37°C under aerobic conditions on a shaker at 150 rpm, the filters were extracted and rinsed with sterile PS to remove unattached cells. In order to determine the CFU number in biofilms, the filters were transferred to a test tube with 5 mL of sterile PS, homogenized with a glass rod, stirred with a Vortex mixer (VWR, United States) for 1 min to disperse the biofilms, and plated on petri dishes to count the CFUs. Filter fibers served as an abrasive material that facilitated the destruction of cell aggregates in the biofilms. Some of the filters with biofilms were washed and used to measure the cells metabolic activity. The optical density (OD) of the planktonic culture was concomitantly measured at  $\lambda = 580$  nm; each planktonic culture sample was plated to count the CFUs. The colonies of the two species on petri dishes differed in color: *S. aureus* MFP03 and *S. epidermidis* MFP04 produced yellowish-white and off-white colonies, respectively. The results of CFU enumeration were plotted on diagrams as CFU number logarithms and as the CFU increment in the form of changes in

the decimal logarithm values ( $\Delta\log$ ), i.e., the difference between the original CFU number in the inoculum volume placed in the test tube and the CFU number in the liquid phase (for planktonic cultures) and on the filter (for biofilms). Analysis of the CFU number and its increment made it possible to determine the total cell number in planktonic cultures and mature biofilms and to elucidate the pattern of strain–strain interaction.

**Determination of the metabolic activity** was carried out using the previously described method (Plakunov et al., 2016). The filters were placed into the wells of a 24-well immunological plate. Each well was then filled with 2 mL of 0.2% solution of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), a metabolizable dye that accepts electrons from cellular NADPH oxidoreductases and is converted into insoluble blue formazan. The filters were stained for 30 min at room temperature and thereupon washed with distilled water to remove the dye solution. DMSO (2 mL) was then added to each filter to extract formazan. The OD of the solution was measured at  $\lambda = 595$  nm. The OD value of the formazan extract correlates well with the number of metabolically active cells in the biofilm (Plakunov et al., 2016) and, therefore, was regarded as a cell viability criterion to be used for detecting the possible toxic effects of NUPs.

**Statistical treatment of the data.** At least three repeats of all experiments were conducted. Non-parametric statistics based on the non-parametric Mann–Whitney test was used to verify the validity of the differences between the results to be compared. Differences with  $p < 0.05$  were considered as valid; they are marked with asterisks on the figures. Computation and graph construction procedures were carried out using the Microsoft Office Excel 2007 software package.

## RESULTS AND DISCUSSION

According to the results of the studies presented on Fig. 1, the growth constants of the monospecies cultures of *S. aureus* MFP03 and *S. epidermidis* MFP04 were almost identical. They were 0.6 and 0.69 h<sup>−1</sup>, respectively, in the control system at 37°C; the doubling times were 61 and 69 min, respectively. The death rate constants were 0.009 and 0.05 h<sup>−1</sup>, respectively, in the control system at 37°C. The maximum optical density values of *S. aureus* MFP03 and *S. epidermidis* MFP04 at 37°C were also quite similar; they were located within the 0.9–1 OD<sub>580</sub> range (the data on *S. aureus* are not shown). NUP had no appreciable effect on the growth parameters of both cultures. Only in the case of *S. epidermidis* addition of ANP at a concentration of 10<sup>−6</sup> M slightly increased the doubling time (to 89 min) and decreased the growth rate constant to 0.63 h<sup>−1</sup> and the death rate constant to 0.06 h<sup>−1</sup>. The lag phase duration at 37°C

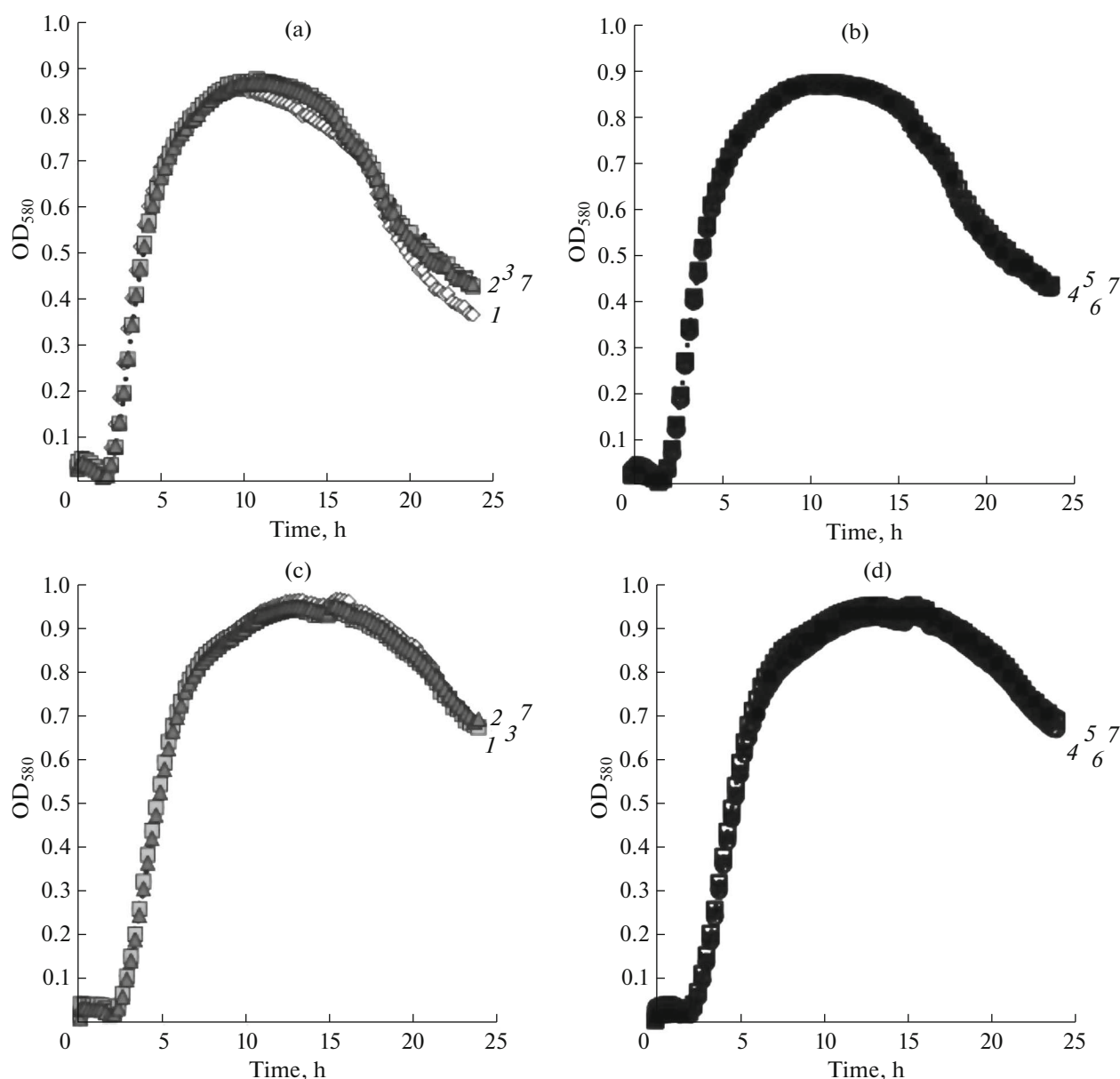
remained virtually unchanged in the presence of NUPs; it was 1 h with *S. aureus* MFP03 and 1.8 h with *S. epidermidis* MFP04.

At 33°C, a somewhat different pattern was observed. The growth rate constants in the control were 0.63 and 0.69 h<sup>−1</sup> for *S. aureus* MFP03 and *S. epidermidis* MFP04, respectively; the doubling times were 63 and 67 min, respectively. The control death rate constant of *S. epidermidis* MFP04 at 33°C was 0.04 h<sup>−1</sup>. In *S. aureus* MFP03, the onset of the death phase was not observed during the 24 h of incubation. The lag phase duration was somewhat increased compared to that at 37°C; it was 1.5 h for *S. aureus* MFP03 and 2 h for *S. epidermidis* MFP04. The maximum OD values were within the 0.9–1.0 range, like those at 37°C. The growth parameters of both microorganisms were not affected by the addition of NUPs, regardless of their concentrations. This was quite consistent with the results of the earlier work that was carried out with other microorganisms (Veron et al., 2008; Rosay et al., 2015).

Both NUPs appreciably influenced development of the monospecies biofilms of the tested bacteria, and the pattern of this interaction significantly varied depending on the cultivation conditions (Fig. 2).

The growth of *S. epidermidis* MFP04 biofilms at 37°C was stimulated by NUPs (Fig. 2a): the maximum effect of ANP was attained at a concentration of 1 × 10<sup>−7</sup> M; the maximum growth level was 129% of that in the control system. CNP at all tested concentrations somewhat increased biofilm growth. The most prominent stimulation occurred at a concentration of 1 × 10<sup>−6</sup> M: the growth level was 115% of that in the control system. In contrast, the growth of *S. aureus* MFP03 biofilms was suppressed by both NUPs (Fig. 2b). ANP exerted the strongest inhibitory effect on the growth of *S. aureus* MFP03 biofilms at a concentration of 1 × 10<sup>−7</sup> M: the growth level was 71% of that in the control system. CNP maximally suppressed growth at a concentration of 1 × 10<sup>−6</sup> M: it was 80% of that in the control system.

A decrease in cultivation temperature to 33°C resulted in reversing the NUP effect on biofilm growth of both microorganisms: *S. epidermidis* MFP04 formed biofilms less efficiently than in the control system (Fig. 2c). The effect was small with ANP, and its amplitude was approximately the same within the whole tested concentration range; the growth level was about 84% of that in the control system. CNP produced a stronger inhibitory effect on biofilm growth, especially at a concentration of 1 × 10<sup>−8</sup> M, when the growth level was about 73% of that in the control system. As for *S. aureus* MFP03 (Fig. 2d), a decrease in ANP concentration resulted in a stimulatory effect. The maximum stimulation was attained at 1 × 10<sup>−8</sup> M; the growth level was 112% of that in the control system. CNP also statistically significantly stimulated biofilm growth at all tested concentrations; the maximum



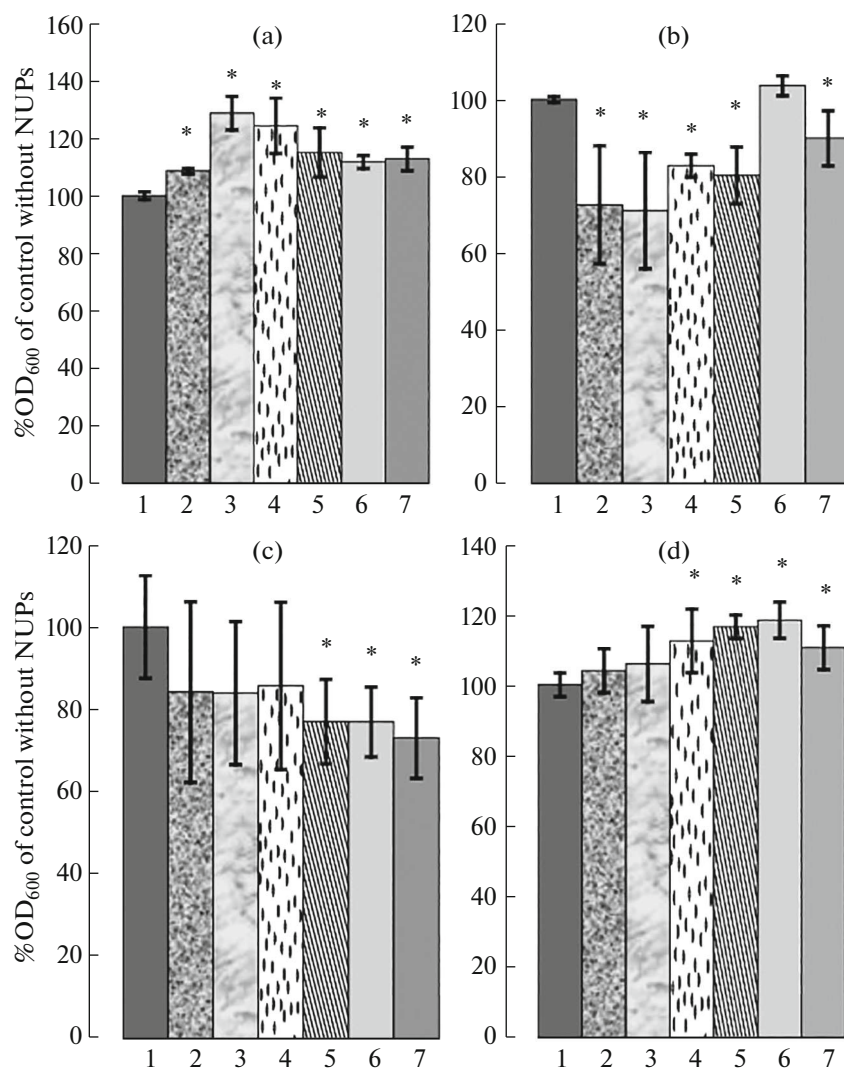
**Fig. 1.** Effects of ANP and CNP on the growth of *S. epidermidis* MFP04 cultures in relation to the cultivation conditions. Growth curve parameters: (a) growth with ANP at 37°C; (b) growth with CNP at 37°C; (c) growth with ANP at 33°C; (d) growth with CNP at 33°C. Markers: (1) ANP  $1 \times 10^{-6}$  M; (2) ANP  $1 \times 10^{-7}$  M; (3) ANP  $1 \times 10^{-8}$  M; (4) CNP  $1 \times 10^{-6}$  M; (5) CNP  $1 \times 10^{-7}$  M; (6) CNP  $1 \times 10^{-8}$  M; (7) control system without NUPs. \*,  $p < 0.05$ .

stimulation was attained at  $1 \times 10^{-7}$  M; the growth level was 111% of that in the control system. Analysis of the data on CV-stained biofilms and on the culture growth dynamics supports the suggestion that NUP-induced growth dynamics changes are predominantly due to attenuation of biofilm formation and, accordingly, a decrease in light scattering by biofilms growing on plate well bottoms.

The results obtained with CV-stained biofilms were confirmed by analyzing the CLSM data (Figs. 3 and 4). The biomass density (Figs. 3a, 1; 3b, 1) and the average biofilm thickness (Figs. 3c, 1; 3d, 1) of *S. epi-*

*dermidis* MFP04 significantly increased in the presence of NUPs at 37°C. The biomass density was  $23.52 \mu\text{m}^3/\mu\text{m}^2$  and the average thickness was  $24.7 \mu\text{m}$  in the control system. Addition of ANP at all tested concentrations resulted in an increase in biomass density; the maximum increase occurred at a concentration of  $10^{-6}$  M; the biomass density was  $29 \mu\text{m}^3/\mu\text{m}^2$ , i.e., 125.8% of that in the control system. The average thickness also increased, and the maximum increase occurred at a concentration of  $10^{-6}$  M; the biofilm thickness was  $30.3 \mu\text{m}$ , i.e., 123% of that in the control system. CNP exerted a similar stimulatory effect at



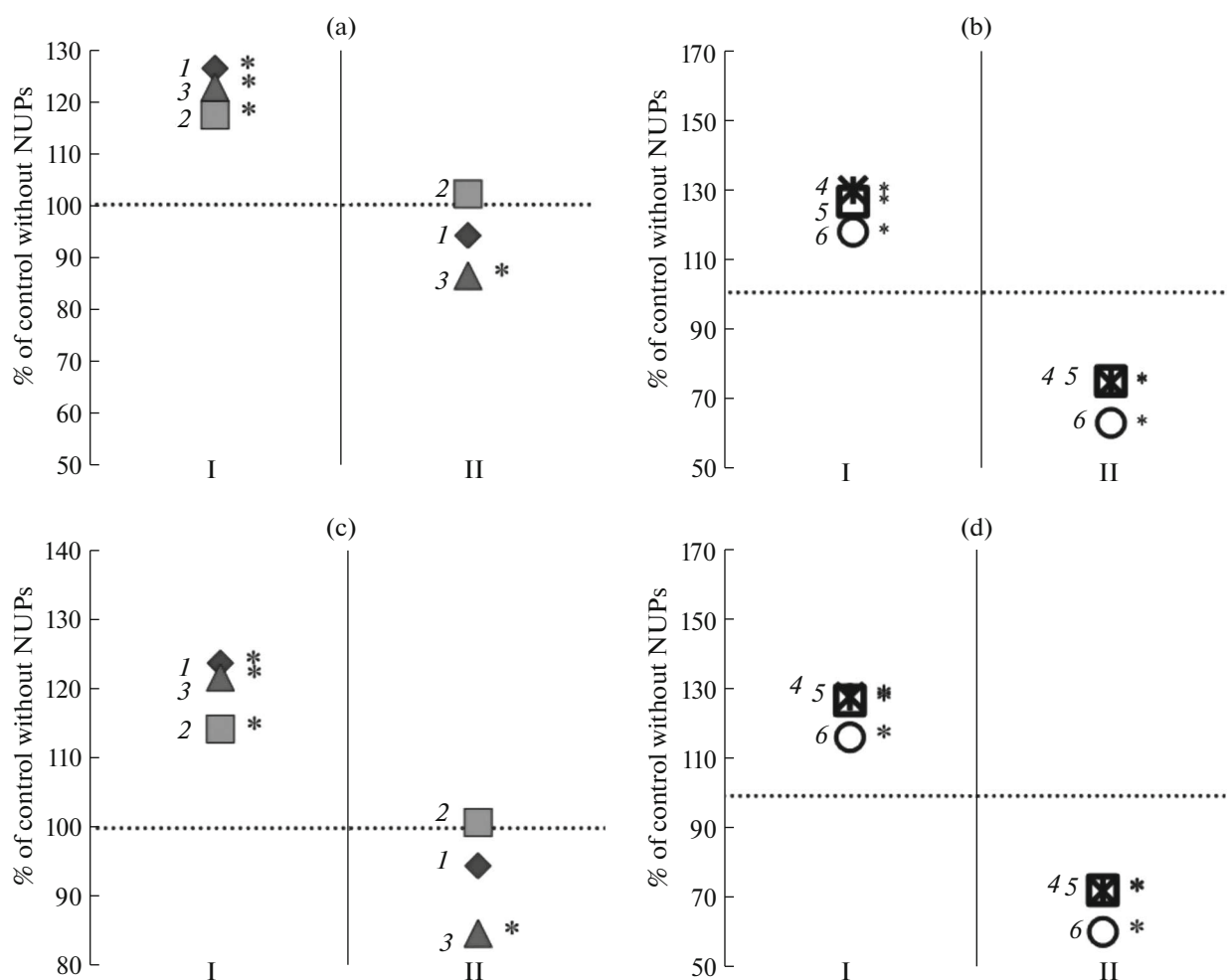


**Fig. 2.** Effects of ANP and CNP on the growth of the monospecies biofilms of *S. epidermidis* MFP04 and *S. aureus* MFP03. CV staining. (a) *S. epidermidis* MFP04 biofilms at 37°C; (b) *S. aureus* MFP03 biofilms at 37°C; (c) *S. epidermidis* MFP04 biofilms at 33°C; (d) *S. aureus* MFP03 biofilms at 33°C. Marker designations (1–7): see Fig. 1.

37°C: the biomass density and the average biofilm thickness maximally increased at a CNP concentration of  $10^{-7}$  M; they were  $30.3 \mu\text{m}^3/\mu\text{m}^2$ , i.e., 129% and  $31.7 \mu\text{m}$ , i.e., 128%, respectively).

A decrease in *S. epidermidis* MFP04 cultivation temperature to 33°C resulted in reversing the NUP effect that became inhibitory (Figs. 3a–3d; 2). The biomass density and the average biofilm thickness in the control system were  $16.83 \mu\text{m}^3/\mu\text{m}^2$  and  $16.93 \mu\text{m}$ , respectively. Upon addition of ANP, both parameters were observed to change; the changes were maximally

significant at an ANP concentration of  $10^{-8}$  M; the biomass density was  $14.5 \mu\text{m}^3/\mu\text{m}^2$ , i.e., 86% of the control level, and the average thickness was  $14.4 \mu\text{m}$ , i.e., 84.9% of the control level). CNP also decreased the biomass density and the average biofilm thickness in *S. epidermidis* MFP04. The maximum effect was attained at a concentration of  $10^{-7}$  M: the biomass density decreased to  $12.5 \mu\text{m}^3/\mu\text{m}^2$ , i.e., 74.2% of the control level and the average thickness to  $10.31 \mu\text{m}$ , i.e., 60.9% of the control value.



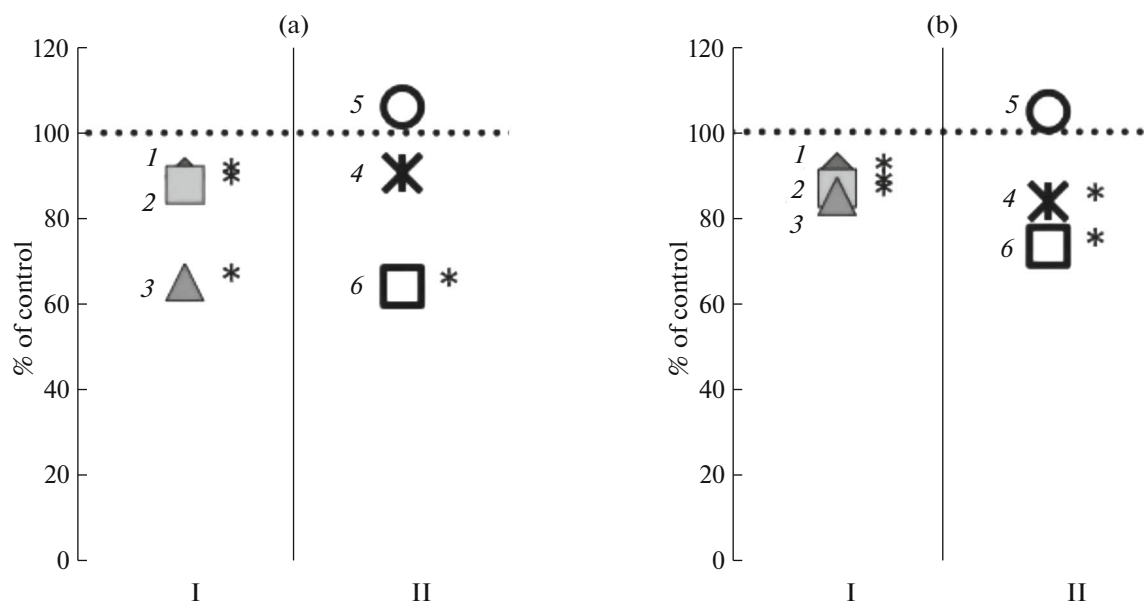
**Fig. 3.** Confocal laser scanning microscopy: the effects of ANP and CNP on the monospecies biofilms of *S. epidermidis* MFP04 under various cultivation conditions. Control, (a) biofilm without NUPs (dashed line, 100%). Biofilm characteristics: a, changes in biofilm biomass density with ANP; (b) changes in average biofilm thickness with CNP; (c) changes in biofilm biomass density with ANP; (d) changes in average biofilm thickness with CNP. Cultivation conditions: I, 37°C, II, 33°C. Marker designations (1–6): see Fig. 1; dashed line, control without NUPs. \*,  $p < 0.05$ .

A diametrically opposite trend occurred in *S. aureus* MFP03 at 37°C: the average biofilm thickness and the biofilm biomass density decreased in the presence of NUPs (Fig. 4). The average thickness in the control system was 30.13  $\mu\text{m}$ , and the biomass density was 27.66  $\mu\text{m}^3/\mu\text{m}^2$ . The strongest inhibitory effect of ANP was observed at a concentration of  $10^{-8}$  M: the average thickness and the biomass density decreased to 25.6  $\mu\text{m}$ , i.e. to 85% of the control value, and to 18  $\mu\text{m}^3/\mu\text{m}^2$ , i.e. to 65% of the control value.

The opposite effects exerted by the peptides, under the same conditions, on closely related microorganisms with distinct functional properties and roles in the human skin testify to a potentially important function of NUPs as skin microbiota composition regulators. Apart from humoral phenomena in the human

organism, they may be implicated in protective and immunological processes. Alterations in biofilm structure and thickness in the presence of the tested NUPs are clearly visible on the three-dimensional images of SYTO9-stained *S. epidermidis* MFP04 biofilms obtained by us (Fig. 5).

In the control system at 37°C, the *S. epidermidis* MFP04 biofilm was formed as a dense layer with a smooth surface without significant irregularities and gaps (Fig. 5a). After adding ANP (Fig. 5b) and CNP (Fig. 5c), the biofilm thickness and biomass density increased, but the visual features remained unchanged: there was a dense biomass layer without gaps. After decreasing the temperature to 33°C, *S. epidermidis* MFP04 biofilms were less developed than at 37°C (Fig. 5d): they were looser and displayed gaps (dark nonfluorescent areas). Addition of ANP (Fig. 5e) and CNP (Fig. 5f) further deteriorated the



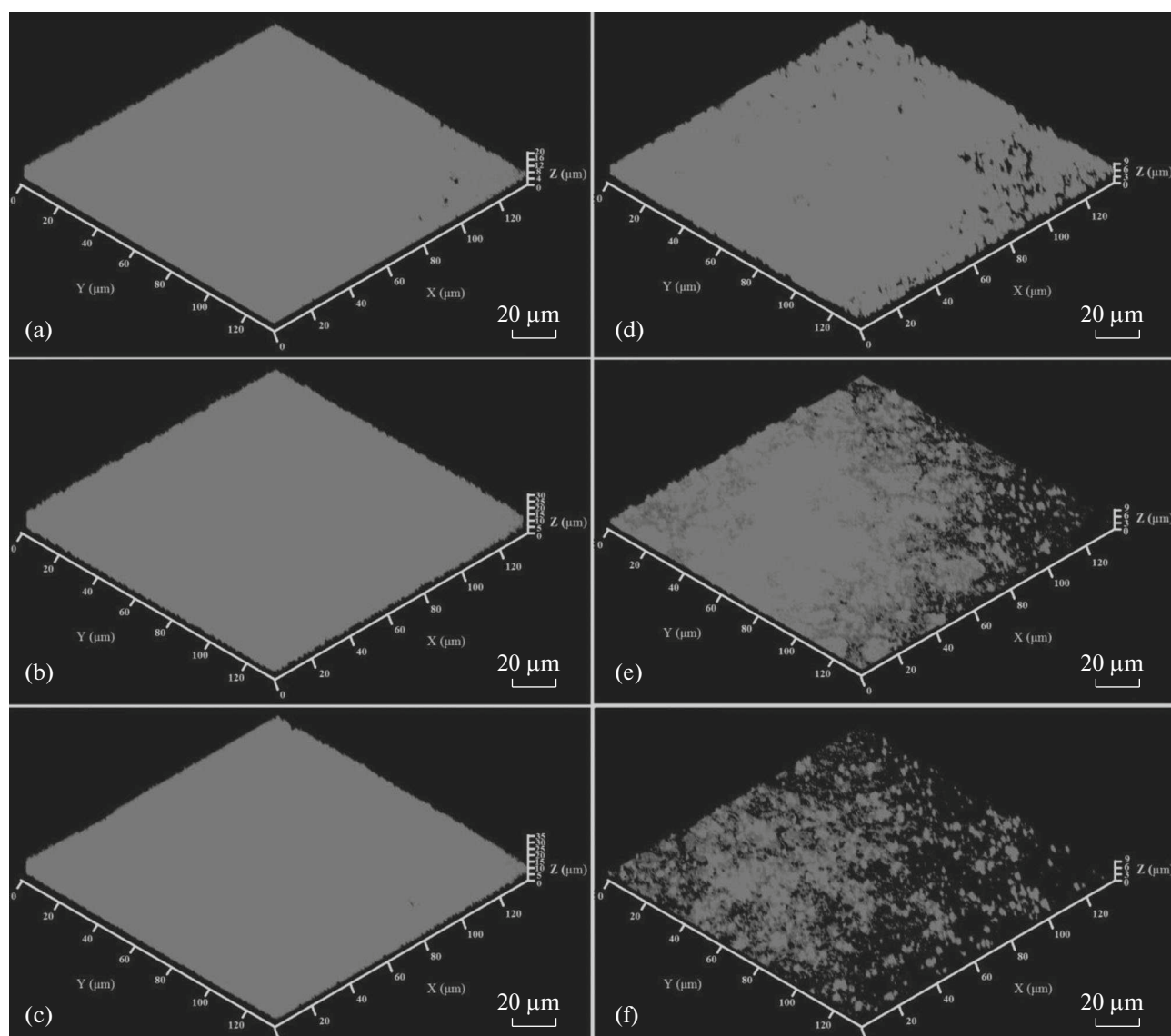
**Fig. 4.** Effects of ANP and CNP on the growth of the monospecies biofilms of *S. aureus* MFP03 at 37°C. Control, the biofilm without NUPs (dashed line, 100%). Biofilm characteristics: (a) biofilm density changes; (b) average thickness changes. The NUPs added: I, ANP, II, CNP. Marker designations (1–6): see Fig. 1. \*,  $p < 0.05$ .

biofilm growth pattern; in the presence of CNP, they actually represented conglomerations of microcolonies with large empty areas between them.

Since each microbial component in multispecies (binary) biofilms may significantly affect the metabolism of the other component(s), the effect of NUPs on the *S. aureus* MFP03 and *S. epidermidis* MFP04 binary biofilms was investigated. Comparative analysis of the CFU number increase in the binary biofilms of the tested staphylococci revealed that *S. aureus* strain MFP03 possessed a considerable competitive advantage over *S. epidermidis* strain MFP04 both in the mixed planktonic culture and in the binary biofilms (Fig. 6). Upon an average,  $5 \times 10^7$  cells/100  $\mu$ L ( $\log = 7.7$ ) were added to the binary and monospecies systems. The CFU number in *S. aureus* MFP03 monospecies biofilms was  $3.57 \times 10^9$  cells ( $\log = 9.55$ ) after 24 h of cultivation in the control system. The CFU number increment  $\Delta \log$  was 1.8 (Fig. 6a). The CFU number was  $2.56 \times 10^{10}$  ( $\log = 10.41$ ) in the monospecies planktonic cultures. The CFU number increment was 2.66. In binary biofilms, the CFU number of *S. aureus* MFP03 was  $3.59 \times 10^9$  ( $\log = 9.81$ ), and its CFU number increment was 1.78 (Fig. 6a). The average CFU number of *S. aureus* MFP03 in binary planktonic cultures was  $1.9 \times 10^{10}$  ( $\log = 10.28$ ) in the control system, and the CFU number increase  $\Delta \log$  was 2.53 (Fig. 6b). After 24 h of incubation, the control CFU number of *S. epidermidis* MFP04 was  $2.41 \times 10^9$  ( $\log = 9.38$ ) in the monospecies biofilms; accordingly, the CFU number increment was 1.63 (Fig. 6a; 1). The control CFU number in the monospecies planktonic

cultures of *S. epidermidis* MFP04 was  $1.55 \times 10^{11}$  ( $\log = 11.2$ ), and the CFU number increment was  $\Delta \log = 3.45$ . The control CFU number of *S. aureus* MFP03 in binary biofilms was  $3.46 \times 10^9$  ( $\log = 9.54$ ), and the CFU number increment  $\Delta \log$  was 1.79. The average CFU number of *S. epidermidis* MFP04 in binary biofilms was  $1.72 \times 10^8$  ( $\log = 8.24$ ). Hence, the CFU number increment decreased to 0.49. The control CFU number of *S. epidermidis* MFP04 in the planktonic cultures was  $1.58 \times 10^{10}$  ( $\log = 10.2$ ), and the CFU number increment was 2.45 (Fig. 6b; 2). Therefore, the CFU number difference between the monospecies and binary planktonic cultures or biofilms of *S. epidermidis* MFP04 reached one order of magnitude in all tested systems.

Addition of the tested NUPs to the nutrient medium produced an effect that was supportive of the results of the previous studies. The CFU number increment in the monospecies biofilms of *S. epidermidis* MFP04 increased at ANP and CNP concentrations of  $1 \times 10^{-8}$  M ( $\Delta \log = 2.02$  and 1.81, respectively), whereas high concentrations of these peptides had no significant effect. The monospecies planktonic cultures of *S. epidermidis* MFP04 were insensitive to the NUPs: the CFU number increment remained at the control level. However, the CFU number in the monospecies biofilms of *S. aureus* MFP03 significantly decreased in the presence of the NUPs in all tested systems. The  $\Delta \log$  value was 1.23–1.41, depending on the concentration, whereas planktonic cultures were insensitive or insignificantly sensitive to the NUPs. No changes in the pattern of the NUP effect on *S. aureus* MFP03 and its strength occurred in any

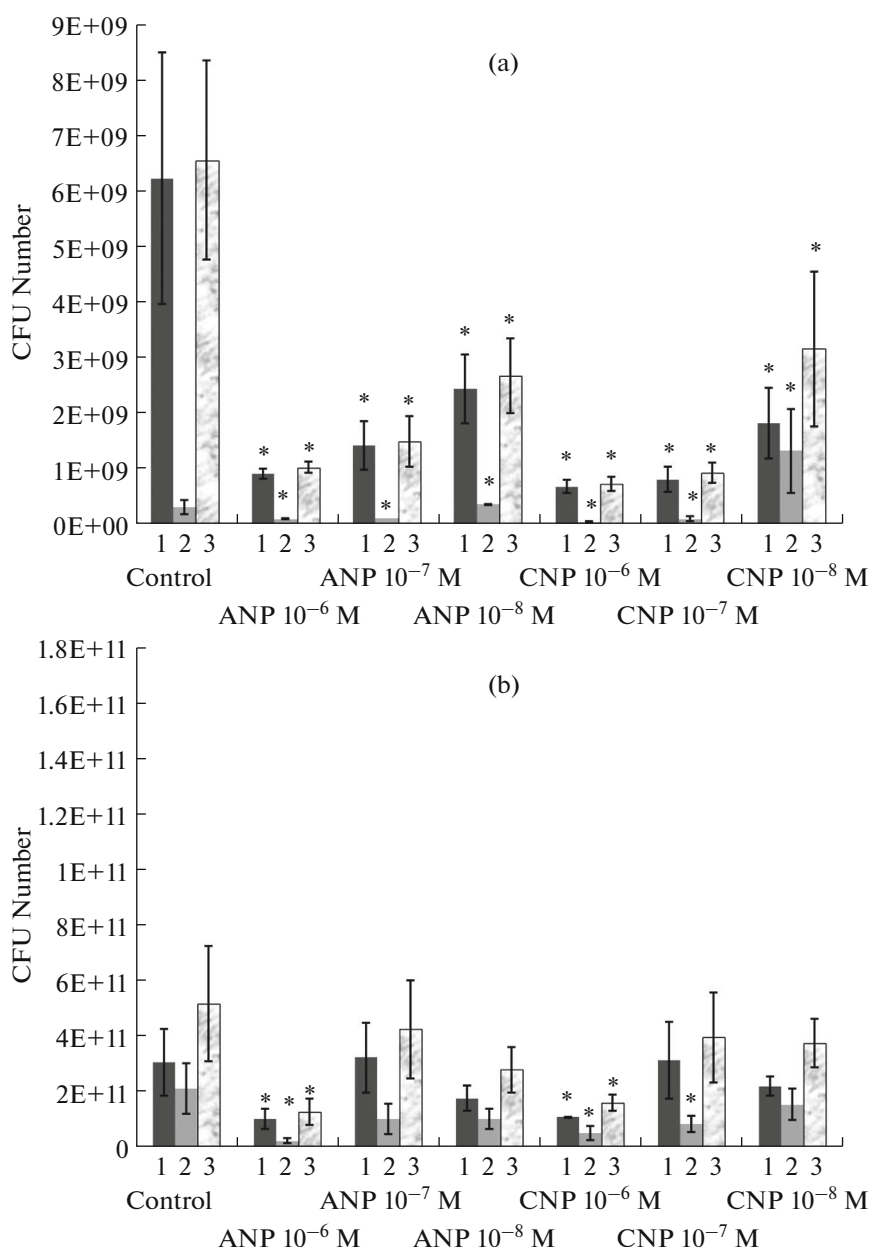


**Fig. 5.** CLSM images of the monospecies biofilms of *S. epidermidis* MFP04 that were grown with NUPs and stained with SYTO9: (a) control at 37°C; (b) ANP  $1 \times 10^{-8}$  M at 37°C; (c) CNP  $1 \times 10^{-7}$  M at 37°C; (d) control at 33°C; (e) ANP  $1 \times 10^{-8}$  M at 33°C; (f) CNP  $1 \times 10^{-7}$  M at 33°C.

of the systems (Figs. 6c–6d; 2). Addition of NUPs at all tested concentrations decreased the CFU numbers of both species in the binary system. However, the NUP concentrations that increased the CFU number increment in the monospecies biofilms of *S. epidermidis* MFP04, also increased the CFU number increment of *S. epidermidis* MFP04 in the binary biofilms. CNP at a concentration of  $1 \times 10^{-8}$  M augmented the CFU number increment by  $\Delta \log = 0.46$ , compared to the control system (the control CFU number was  $3.2 \times 10^9$ ; it became  $1.34 \times 10^{10}$  after adding CT3). ANP applied at the same concentration changed the  $\Delta \log$  value by 0.08, but this effect was to be regarded as insignificant. The NUP concentrations that produced no considerable effect on the monospecies biofilms of

*S. epidermidis* MFP04 also exerted only an insignificant influence on the binary system. By decreasing the CFU numbers of *S. aureus* MFP03, the NUPs shifted the balance in the biofilms in favor of *S. epidermidis* MFP04, and this trend was particularly prominent at a CNP concentration of  $1 \times 10^{-8}$  M. The NUP concentrations that stimulated the growth of monospecies biofilms failed to affect planktonic cultures. The NUP concentrations that had no effect on the monospecies biofilms somewhat decreased the CFU number increment of *S. epidermidis* MFP04 in the binary culture.

The NUP effects on CFU numbers correlated with the optical density of the formazan extracts from monospecies and binary biofilms (Fig. 7).



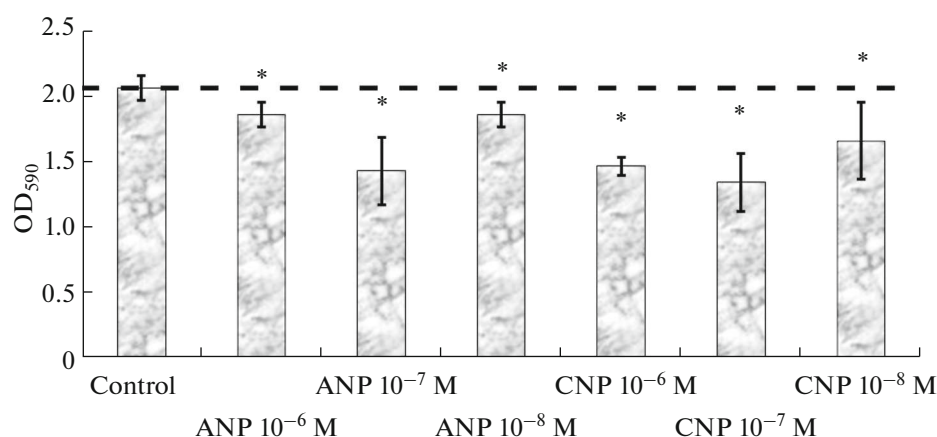
**Fig. 6.** CFU numbers in binary biofilms (a) and planktonic cultures (b) of *S. aureus* MFP03 and *S. epidermidis* MFP04. 1, CFU number of *S. aureus*; 2, CFU number of *S. epidermidis*; 3, total CFU number in binary biofilms. \*,  $p < 0.05$ .

The optical density of the formazan extract and, therefore, the total metabolic activity significantly decreased due to the decrease in CFU number after adding the NUPs. The average control OD value was 2.1; it maximally decreased when either of the NUPs was added at a concentration of  $1 \times 10^{-7}$  M. The OD values became 1.42 and 1.33 after addition of ANP and CNP, respectively. The decrease in OD of the extracts was due to a decrease in the CFU number of the components of the binary biofilms, predominantly, of *S. aureus*, since the *S. epidermidis* contribution was

relatively small. According to the data on MTT-stained samples, the metabolic activity of monospecies biofilms correlated with the CFU number (data not shown): a decrease in CFU number resulted in a decrease in the optical density of the formazan extract.

Our studies revealed that *S. aureus* MFP03 possesses a considerable competitive advantage over *S. epidermidis* MFP04 both in the planktonic co-culture and binary biofilms, in which the advantage is especially pronounced. These results may account for the fact that *S. aureus* quickly develops into the domi-





**Fig. 7.** Effects of NUPs on the metabolic activity of the cells in the binary biofilms of *S. aureus* MFP03 and *S. epidermidis* MFP04. \*,  $p < 0.05$ . Horizontal axis, the optical density of formazan extract.

nant species during various skin diseases in which the skin cover is infected by this bacterium.

This is the first report on the effect of the A and C types of human NUPs on skin microorganisms and their interaction in binary systems. Importantly, NUPs do not seem to function in the well-known capacity of human organism-produced antibacterial substances. In all likelihood, they play the part of communication signals. Apart from communication among human cells, they may be involved in the interaction between the human organism and the skin microbiota. Evidence for this suggestion is provided by the fact that the peptides only produce a weak effect on the growth of planktonic cultures but affect the formation of monospecies biofilms and the competitive advantages of microorganisms in binary biofilms and planktonic cultures.

Of special relevance seems to be the fact that, at 37°C, ANP and CNP stimulate the growth of *S. epidermidis* monospecies biofilms but suppress that of the monospecies biofilms of *S. aureus* MFP03. This fact enables us to put forward a hypothesis concerning the regulatory and plausibly the protective role of NUPs in the human skin. Under normal conditions, i.e., when the skin is not inflamed and its temperature is approximately 30 to 33°C, depending on the skin area involved, the growth of the *S. epidermidis* biofilm or planktonic culture is slightly suppressed or unaffected by NUPs. When the balance among skin microorganisms is disrupted, the significantly more dangerous and aggressive *S. aureus* starts to intensely proliferate. This results in inflammation and, accordingly, in a rise in local or systemic temperature up to 37°C and above. In this situation, natriuretic peptides suppress the growth of *S. aureus* and stimulate that of *S. epidermidis*. This mitigates the risk of complications and of a further deterioration of the patient's health state caused by excessive *S. aureus* proliferation; the normal bal-

ance among skin microorganisms is reestablished. This may account for the protective function of *S. epidermidis* with regard to the human skin. This idea seems to be supported by an increasing body of evidence (Iwase et al., 2010, Sugimoto et al., 2013), assuming that *S. epidermidis* is aided by the humoral systems of the human organism that combat aggressive *S. aureus* strains. This hypothesis is speculative; additional studies including those conducted with in vivo systems are necessary. Nevertheless, the results of our research give sufficient grounds for considering NUPs an important component of the interactive skin microbiota—human organism system. This opens up new prospects in terms of the basic aspects of dermatology and microbiology as well as practical developments in cosmetology and pharmaceuticals. Our results provide evidence for a close evolutionary relationship between the human organism and the microbial commensals of its skin.

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*Translated by A. Oleskin*



**Regulation of monospecies and mixed biofilms formation of skin**

***Staphylococcus aureus* and *Cutibacterium acnes* by human natriuretic peptides**

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**Keywords:** *Staphylococcus aureus*, *Cutibacterium acnes*, biofilms, multispecial biofilms, natriuretic peptides, regulation, skin microbiome, human skin-microbiome interaction.

**Short title :** Effect of human natriuretic peptides on *Staphylococcus aureus* and *Cutibacterium acnes* biofilms

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### ABSTRACT

*Staphylococcus aureus* and *Cutibacterium acnes* are common representatives of human skin microbiome. However, these bacteria organized in biofilm could be involved in a number of skin disorders such as acne or psoriasis. They inhabit hollows of hair follicles and skin glands in which they form biofilms. Here, there are continuously exposed to human hormones including the human natriuretic peptides (NUPs). We first observed that the atrial natriuretic peptide (ANP) and the C-type natriuretic peptide (CNP) have a strong effect on the skin *S. aureus* and *C. acnes* biofilm formation. These effects are significantly dependent of the aero-anaerobic conditions and of the temperature. We also showed that both ANP and CNP increased competitive advantages of *C. acnes* towards *S. aureus* in mixed biofilm. Because of their temperature dependent effects, NUPs appear to act as thermostat allowing the skin to modulate bacterial development in normal and inflammatory conditions. This is an important step to understanding of how human neuroendocrine systems can regulate the cutaneous microbial community and should be important for application in fundamental sciences, medicine, dermatology and cosmetology.

## INTRODUCTION

Skin microorganisms that compose the skin microbiome form as a complex community in which all members are closely interrelated (Prescott et al., 2017; Belkaid et al., 2014). This community evolved with humanity and became an essential part of a human body (Chiller et al., 2001). *Staphylococcus aureus* and *Cutibacterium acnes* are main actors of the human skin microbiome, that prefer sebum-rich microniches such as skin glands hollows and hair follicles (Grice et al. 2009; Aubin et al., 2014; SanMiguel et al., 2015; Feuerstein et al., 2017). Both *S. aureus* and *C. acnes* are opportunistic pathogens, and sometimes can be at the origin of skin disorders. In this way, *C. acnes* is considered as one of acne vulgaris causes (Fitz-Gibbon et al., 2013; Achermann et al., 2014; Pettersson et al., 2018). In parallel, it has been shown that *C. acnes* can be a cause of complications after operations and prosthetic implants setup (Howlin et al., 2017). Thus far, the role of *S. aureus* in acne vulgaris remains unclear (Krovash et al., 2012; Totte et al., 2016). However, it was shown that *S. aureus* colonizing of acne lesions that can strengthen inflammation (Totte et al., 2016; Dreno et al., 2017). Moreover, *S. aureus* can be responsible of complications and inflammation during psoriasis and skin wounds (Elfatoiki, 2016; Lacey et al., 2016; Totte et al., 2016). Both bacteria are able to form biofilms inside niches that they colonize (Daum, 2007; Jahns et al., 2012; Jahns & Alexeyev, 2014). Biofilms are microbial communities embedded in extracellular polymeric matrix, conferring to bacterial cells included in these structures a protection against unfavorable environmental factors (Nikolayev & Plakunov, 2007). Multispecies biofilms are the most common form of microbial life on Earth (Nozhevnikova et al., 2015), and human skin should not be an exception. Probably, multispecies microbial biofilms including those formed by both *C. acnes* and *S. aureus*, can be a cause of acne vulgaris and other skin disorders, because such biofilms are formed inside comedones and hair follicles (Ten Broeke-Smits et al., 2010; Khorvash et al., 2012; Jahns et al., 2012; Matard et al., 2013; Jahns & Alexeyev, 2014).

There are numerous evidences that the microbial community of skin is not just closely related with human organism, but exchanges information with its host. Those connection seem to be, at least

partly dependent of molecules which are not classically considered as bacteria effectors, such as hormones, neuropeptides and neurotransmitters. For instance, it was shown that *Escherichia coli* possess several adrenaline receptor/sensors. Those catecholamines (adrenaline and noradrenaline) are able to regulate a number of processes in bacteria such as toxin synthesis, growth and pili synthesis (Hughes & Sperandio, 2008). Near to neurotransmitters, peptidic hormones are also able to modulate bacterial physiology (Lesouhaitier et al., J. Innate Immun. (in press)). In this way, one of most abundant neuropeptide of skin – substance P, which is released by skin nerve endings and diffuses in sweat and skin (Feuilloley, 2018) is exerts a central role in virulence expression in cutaneous strains of *S. aureus* and *Staphylococcus epidermidis*. In *S. aureus*, substance P increases staphylococcal enterotoxin C2 synthesis, an in *S. epidermidis* the whole biofilm formation activity (N'Diaye et al., 2016). Conversely, at high concentrations, substance P and many other neuropeptides such as neuropeptide Y, can exert antimicrobial activities against very different bacterial species including *E. coli*, *Enterococcus faecalis* or *Lactobacillus acidophilus* (Karim et al., 2008).

Recently, Natriuretic peptides (NUP) were of especial interest in the specific field of microbial endocrinology as designated by Lyte (2004). To date NUP are represented by three main members: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (Potter et al., 2009). Among these peptides, it has been observed that CNP is the more active on *Pseudomonas aeruginosa*. CNP was shown to modulate toxins and lipopolysaccharide synthesis in *P. aeruginosa* (Veron et al., 2007; Blier et al., 2011). In parallel, it has been observed that CNP increases *P. aeruginosa* and *P. fluorescens* cytotoxicity (Veron et al., 2007; Veron et al., 2008), as well as *P. aeruginosa* virulence in a *Caenorhabditis elegans* model (Blier et al., 2011). Interestingly, it was demonstrated that all these effects are specific and are consecutive to the binding of this human hormone on a bacterial sensor named AmiC which is an ortholog of C-type NUP receptor (Rosay et al., 2015). NUPs are synthesized by different human cells including heart (atrium) cardiomyocytes and blood vessels endothelial cells. Transported by the blood and produced locally by the capillary walls, they could impact the human skin microbiota in areas particularly rich in capillary vessels. In this

95 regard, the bulge of the hair follicle is a region of special interest because of the presence of a dense  
96 network of capillary vessels at the immediate vicinity of the hair follicle hollow (Xiao et al., 2013)  
97 where gland ducts open and a rich microbial community, including *C. acnes* and *S. aureus*, develops  
98 (Lange-Asschenfeldt et al., 2011; Matard et al., 2013). So, these two bacteria in this area are under  
99 permanent exposure of NUPs, that potentially can affect bacterial growth and biofilm formation, and  
100 through this – on general skin status. Recently we have shown that ANP and CNP affect the growth of  
101 binary biofilms of *S. aureus* and *S. epidermidis* (Gannesen et al., 2018). This work is aimed to  
102 investigate ANP and CNP impact on monospecies and binary biofilms of *S. aureus* and *C. acnes*. This  
103 should enlarge our understanding of skin-microbiome interactions.

## MATERIALS AND METHODS

### Strains and cultivation

*Staphylococcus aureus* MFP03 was isolated from skin of the healthy volunteer and was fully characterized (Hillion et al., 2013). The strain was stored at -80°C. For bacterial culture, an aliquot of bacteria was mixed in 25 ml of Luria Bertani medium (LB) and incubated for 24 h at 37°C under agitation.

*Cutibacterium* (former *Propionibacterium*, Scholz and Kilian, 2016) *acnes* strains ribotype 4 (RT4) HL045PA1 and ribotype 5 (RT5) HL043PA2 (acneic strains) initially isolated by Fitz-Gibbon (Fitz-Gibbon et al., 2013) were obtained from BEI Resources American Type Culture Collection (Virginia, United States). These strains are associated to severe forms of acne and differ from non acneic strains (ribotype 6 and in a lower extend ribotypes 1, 2 and 3) by a large plasmide (Locus3) which should confer their virulence properties (Fitz-Gibbon et al., 2013). Bacteria stored at -80°C were initially plated on agar reinforced clostridial medium (RCM). As these strains are strictly anaerobic, the plates were incubated for 72 h in BD GasPack™ under anoxic conditions at 37°C. Colonies were then transferred into sterile conical 50 mL tubes (Falcon) filled to maximal capacity with RCM and grown for 72 h at 37°C.

### Natriuretic peptides

The human atrial natriuretic peptide (ANP) (Alfa Aesar, USA) has a molecular weight of 3080.47 g/mol and the C-type natriuretic peptide (CNP) (PolyPeptide, Strasbourg, France) has a molecular weight of 2196.1 g/mol. Peptides were reconstituted in milli-Q water and stored dissolved at -20°C with molar concentration  $1.623 \times 10^{-4}$  M and  $4.554 \times 10^{-4}$  M for ANP and CNP, respectively. According to literature data about physiological NUP concentrations in human organism (Edwards et al., 1988; Stingo et al., 1992) and data of NUP concentrations used in experiments with eukaryotic cells (Klinger et al., 2013), we studied the effect of the peptides at concentrations ranging from  $10^{-6}$  M to  $10^{-8}$  M and also with mix of two peptides at a concentration of  $10^{-6}$  M. Peptides were added to experimental medium immediately before experiment start.

### Bacterial monospecies cultures growth dynamics

The effects of ANP and CNP on monospecies cultures growth were studied in a Bioscreen C system (Finland) in 100-well flat-bottom microtiter plates Honeycomb (Growth Curves, USA). On the bottom of the well an aliquot of NUP was distributed. Then 200 µl of growth medium were injected in each well. *S. aureus* MFP03 was cultivated in tryptic soy broth (TSB, Sigma) with addition of 0.25% glucose (Sigma). *C. acnes* strains were cultivated in RCM medium. Before plating, bacterial cultures were washed twice with physiological saline (PS) at pH 7.0. Then OD<sub>580</sub> of cell suspension in PS was adjusted to 1.0. Finally, 16 µl of culture were injected into each well containing medium and peptides. Some wells were maintained without culture medium (negative control), and some others without peptides (positive control). *S. aureus* MFP03 was cultivated aerobically at 37°C or 33°C (closer to skin physiological temperature). *C. acnes* was also cultivated at 33°C and 37°C owing that acneic strains naturally develop in anoxic deep skin regions. In the case of *C. acnes*, peripheral wells were filled with a CO<sub>2</sub>-producing solution and prepared in anoxic conditions using GasPack™ system and sealed with parafilm before incubation. Optical density of the cultures was determined automatically every 15 min. Growth curves were determined over a minimum of three independent experiments.

### Confocal laser scanning microscopy of monospecies biofilms

Monospecies biofilms of *S. aureus* MFP03 and *C. acnes* RT5 were obtained in 24-well plates with flat glass bottom (Geiner bio-one, Germany). Biofilms were grown according to Gannesen *et al.*, 2018. Briefly, prepared cultures were washed twice with sterile PS and OD<sub>580</sub> was adjusted to 1.0. In each well 300 µl of cell suspension were added, and plates were incubated 2h at room temperature (time for cell adhesion). *C. acnes* strains were incubated in GasPack® (BD) system in anaerobic atmosphere. In each well, the suspension was removed and wells were washed twice with sterile PS to remove non-attached cells. One well was dried and fixed in order to verify adhesion control condition. 1 ml of medium containing ANP or CNP, or no peptides (positive control) was injected into the wells. To find out probable effect of cultivation conditions on biofilm growth, different conditions for each bacterium were tested, they are summarized in **Table 1**.

**Table 1.** Conditions of monospecies biofilm cultivation for CLSM analysis

Strain	<i>S. aureus</i> MFP03				<i>C. acnes</i> RT4, RT5	
Conditions	37°C, aerobic atmosphere, TSB	33°C, aerobic atmosphere, TSB	33°C, anaerobic atmosphere, TSB	33°C, anaerobic atmosphere, RCM	37°C, anaerobic atmosphere, RCM	33°C, anaerobic atmosphere, RCM

Biofilms were grown for 24 h for *S. aureus* MFP03 and for 72 h for *C. acnes*. In each case, where anaerobic atmosphere was necessary, GasPack® system (BD) was used. After incubation time, biofilms were washed twice with sterile PS and stained with SYTo9 Green fluorescent dye for 20 min. Staining dye was then removed and biofilms were rinsed and fixed with ProLong® Diamond Antifade Mountant (Molecular Probes™). Observations were realized using an LSM710 inverted confocal laser-scanning microscope (Zeiss, Germany). Three-dimensional (3D) images and orthocuts were obtained using Zen® 2009 software. Biofilm thickness was quantified with the same software. Images are representative of the biofilm structure observed in a mean of 20 different fields over a minimum of four independent studies. Biofilm density and thickness were calculated over the same number of observations using Zeiss Zen Image Analysis software for light microscopy (ImageJ software package). Statistical differences were determined using the Mann-Whitney nonparametric test. The differences between experimental and control variants were considered reliable at confidence coefficient > 95% ( $P < 0.05$ ).

#### Study of bacteria interaction in binary biofilms

Binary biofilms were obtained on Petri dishes with agar RCM. Glass fiber filters Whatman GF/F 21 mm in diameter were used as carriers. Cultures were prepared as described above. Sterile filters were placed onto RCM surface, after 20 µl of each culture were dropped onto filter center. Biofilms were incubated for 72 h under anaerobic atmosphere at 37°C or 33°C. At 33°C both *C. acnes* RT5 and *S. aureus* MFP03 were used, and different types of biofilms were constructed and are described in **Table 2**. Before plating of secondary colonizers, all filters with biofilms were replaced on Petri dishes



with fresh agar RCM. Secondary colonizers were plated onto the biofilm as a drop of a culture prepared as described above.

**Table 2.** Types of binary biofilms of *S. aureus* MFP03 and *C. acnes* RT5 at 33°C

Binary biofilm type \ Component	Pre-formed biofilm	Secondary colonizer
Simultaneously growing	None	None
Based on aerobically pre-formed biofilm of <i>S. aureus</i> MFP03	24 h biofilm of <i>S. aureus</i> MFP03, formed under aerobic atmosphere	<i>C. acnes</i> RT5, growth for 72 h under anaerobic atmosphere
Based on anaerobically pre-formed biofilm of <i>S. aureus</i> MFP03	24 h biofilm of <i>S. aureus</i> MFP03, formed under anaerobic atmosphere	<i>C. acnes</i> RT5, growth for 72 h under anaerobic atmosphere
Based on pre-formed biofilm of <i>C. acnes</i> RT5	72 h <i>C. acnes</i> RT5 biofilm, formed under anaerobic atmosphere	<i>S. aureus</i> MFP03, growth for 24 h under anaerobic atmosphere

In parallel, as control conditions monospecies biofilms were grown. Monospecies biofilms were manipulated in the same way as binary biofilms were. All samples were divided into two parts: one was dedicated to cells metabolic activity analysis, while the second one was used for *S. aureus* MFP03 counting. Experiments were conducted independently at least three times. Statistical differences were determined using the Mann-Whitney nonparametric test. The differences between experimental and control variants were considered reliable at confidence coefficient > 95% ( $P < 0.05$ ).

### Determination of cells metabolic activity in biofilms

The metabolic activity of cells in biofilms was determined as described by Gannesen et al., 2018 using the method of Plakunov et al., 2016. Briefly, filters with biofilms were stained with 3-(4,5-dimethyl-2-tiazolyl)-2,5-dyphenil-2H-tetrazolium bromide (MTT). MTT is an acceptor of electrons from electron-transport pathways, when reduced it becomes formazan which is non-soluble in water. The amount of formazan generated is proportional to the metabolic activity of the bacteria. Filters with biofilms were stained with a 0.2% solution of MTT in sterile LB for 30 min. After, filters were washed out from MTT with distilled water, dried, and formazan was extracted with dymethylsulfoxide (DMSO, Sigma). Then OD590 of extracts was measured.

### *S. aureus* MFP03 CFU count in binary biofilms

After biofilms plating the amount of CFU in 20 µl of initial culture was determined by plating on Petri dishes for control. When biofilms were mature, *i.e.* after 24 or 72 h of culture, filters were homogenized in 5 ml of sterile PS: first by glass stick followed by vortexing for 30 s. Glass fibers played the role of additional abrasive material for cell aggregates disintegration allowing a maximal recovery of the bacteria (Tests for *S. aureus* biofilms dispersal and role of glass fibers as abrasive agent were conducted. *Data not shown*). After biofilm dispersal, cell suspensions were diluted and aliquots were plated on tryptic soy agar (TSA, Sigma) Petri dishes supplemented with 0.25% of glucose. Petri plates were incubated at 37°C for 48 h, and CFU numbers were counted. Experiments were repeated independently at least three times.

**Impact of ANP and CNP on binary biofilms of *S. aureus* MFP03 and *C. acnes* RT5**

The general principle of ANP and CNP effects determination studies was based on the method described in Gannesen et al., 2018. Briefly, biofilms were grown on glass fiber filters Whatman GF/F in RCM in 12-well plates (Thermo Scientific, USA). One filter was placed on the bottom of the wells, where 3 ml of RCM containing NUP was previously added. Only concentrations of NUPs with the strongest effect on monospecies biofilms found in CLSM experiments were studied. Samples without NUPs constituted positive controls. After, 50 µl of cell cultures prepared as described above were added. In parallel, monospecies cultures an biofilms of each bacterium were cultivated in the same conditions as controls. Biofilms were incubated at 33°C for 72 h under anaerobic atmosphere (GasPack®). All samples in each experiment were duplicated on two filters: after incubation one filter of each sample type was stained with MTT, and one was processed to CFU count. Experiments were conducted at least in triplicate.

**Fluorescence *in situ* hybridization (FISH) of *S. aureus* MFP03 and *C. acnes* RT5 binary biofilms**

Biofilms were grown in 24-well plates with flat glass bottom (Geiner bio-one, Germany). Three types of binary biofilms were constructed and studied (**Table 3**).

**Table 3.** Variants of binary biofilms for FISH-labeling and CLSM analysis

Component	Pre-formed biofilm	Secondary colonizer
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Binary biofilm type		
Simultaneously grown	None	None
Aerobically pre-formed <i>S. aureus</i> MFP03	Biofilms of <i>S. aureus</i> MFP03, formed for 24 h under aerobic atmosphere in presence of NUPs	<i>C. acnes</i> RT5, which was plated on 24 h biofilm of <i>S. aureus</i> MFP03. Biofilms were incubated for 72 h in presence of NUPs under anaerobic atmosphere.
Pre-formed <i>P. acnes</i>	Biofilms of <i>C. acnes</i> RT5, formed for 72 h in presence of NUPs	<i>S. aureus</i> MFP03, which was plated on 72 h biofilm of <i>C. acnes</i> RT5. Biofilms were incubated for 24 h in presence of NUPs under anaerobic atmosphere.

In case of simultaneously grown biofilms and primary colonizers in pre-formed biofilms, biofilms were obtained as described above. For simultaneously grown biofilms, 300 µl of each culture per well were injected. Simultaneously, grown biofilms were cultivated for 72 h. All biofilms were incubated under anaerobic atmosphere at 33°C in RCM without agitation. In case of biofilms based on pre-formed ones, when incubation time of primary colonizer was over, biofilms were washed twice with sterile PS. After, 1 ml of fresh medium with ANP or CNP was added in each well, and 40 µl of culture of secondary colonizer were added. As in previously described experiments, we studied the most effective concentrations of NUPs found by CLSM in monospecies biofilms. Then binary biofilms were incubated for needful time. In parallel, controls for hybridization were made by preparing monospecies biofilms of each bacteria by the same pathway as binary ones. Positive control was without addition of NUPs. All experiments were made independently in triplicates.

FISH-labeling was performed as described by Nistico et al., 2014. Briefly, when incubation time was over, biofilms in wells were washed twice with sterile PS and fixed with 100% methanol for 30 min at room temperature. Then methanol was removed, biofilms were dried at room temperature overnight. Fixed biofilms were treated with lysozyme (Sigma) solution (0.1 mg/ml in buffer 0.1M Tris-HCl pH 8.0 + 0.05 M EDTA), 200 µL per well. A piece of paper towel soaked with water was placed in hermetic bag of GasPack® system to maintain high humidity and closed plates with biofilms were introduced inside. Lysozyme treatment was conducted at 37°C for 3 h. Subsequently, lysozyme solution was removed gently by pipetting, and 200 µl of lysostaphin solution (Sigma; 10 mg/ml in

Tris-HCl pH 8.0 + 0.01% of sodium dodecylsulfate (SDS) + 20% formamide) was added in each well. Plates were incubated in the same system for 10 min at 37°C. Then, the lysostaphin solution was removed, and wells were washed twice with phosphate buffer pH 7.4. Cell membranes were permeabilized by exposure for 3 min to increasing concentrations of ethanol (50%, 80% and 100%) in phosphate buffer pH 7.4 at room temperature. After treatment wells with biofilms were properly dried at room temperature.

For *C. acnes* RT5 hybridization, a probe 5'-GCCCCAAGATTACACTTCCG-3' (Eurogentec) was used, as described by Poppert et al., 2010. On 5' the probe was marked with fluorescent dye AlexaFluor546. The probe was dissolved in 50 µl of sterile milli-Q water and stored at -20°C before use. For experiments, an hybridization buffer was prepared in 2-ml Eppendorf tube by mixing 360 µl of 5 M NaCl, 40 µl of 1 M Tris-HCl pH 8.0 and 600 µl of formamide (30% of volume). The solution volume was adjusted to 2 ml with milli-Q water, and 2 µl of 10% SDS were added. Tubes with buffer were enveloped in aluminum foil to avoid light flashing. Directly before experiments, 1.5 µl of probe solution were added into the buffer so that the final concentration of the probe reached 50 ng/µl. In each well with biofilms, 200 µl of hybridization mix were added. Plates were quickly closed and placed into GasPack® hermetic bags with wet paper towels inside; bags were closed and immediately enveloped in aluminum foil to avoid any light. Hybridization was conducted for 1 h at 46°C. While it was going on, washing buffer was prepared. In conical 50 ml centrifuge tubes (Corning) 1 ml of 1 M Tris-HCl pH 8.0, 1.02 µl of 5 M NaCl and 500 µl of 0.5 M sodium ethylene diamine tetraacetate (EDTA) were mixed. The volume was then adjusted to 50 ml with sterile milli-Q water, and 50 µl of 10% SDS were added. After hybridization, the liquid was removed from the wells, 1 ml of washing buffer was added in each well, and plates were incubated in darkness at 48°C for 15 min. Then, the liquid was removed and each well was rinsed with milli-Q water and dried at room temperature in darkness. After, 100 µl of DAPI-containing ProLong™ Diamond Antifade Mountant (ThermoFisher) were added in each well to fix biomass and stain it with DAPI. Plates were covered with aluminum foil and incubated at 4°C overnight.

CLSM analysis was conducted in the red and blue modes of fluorescence. For AlexaFluor546 the laser line 488 nm was used. DAPI was used as total biomass of *S. aureus* MFP03 and *C. acnes* RT5 indicator. For every sample, analysis was realized over at least 20 observation, and by scanning of at least 5 the most representative areas. Scanning was performed in red, in blue mode, and simultaneously in red-blue mode. The biomass density of *C. acnes* RT5 and *S. aureus* MFP03 biofilms was studied and calculated using Comstat 2 plug-in of ImageJ to determine the variations of *S. aureus* and *C. acnes* biomass after exposure to ANP or CNP.

### **Protein-BLAST search**

According to Rosay et al., 2015, the amidase operon regulator AmiC of *P. aeruginosa* has been identified as the receptor/sensor of CNP. We searched for proteins of taxons *Propionibacteriaceae* and *Staphylococcaceae* representatives, which could be similar to AmiC of *P. aeruginosa*. After, the search of *C. acnes* amidase homologies in taxons *Pseudomonas*, *P. aeruginosa* and *Staphylococcaceae* was conducted. Protein-BLAST search was made on NCBI website of National institute of health, USA (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>).

### **Statistical analysis**

All experiments were conducted in at least three independent repeats. Statistical significance of results was evaluated by use of non-parametric Mann-Whitney test. Significant difference was marked with \* for  $p$  value  $< 0.05$ .

## RESULTS

### Growth dynamics of *S. aureus* MFP03 and *C. acnes* strains in the presence of NUPs

At 37°C (**Figure 1A**), the growth of *S. aureus* MFP03 ( $0.6\text{ h}^{-1}$ ), generation time (61 min) and death constant after stationary phase  $0.009\text{ h}^{-1}$  were measured. The maximal OD<sub>580</sub> in control conditions reached 0.8 and the mean lag-phase length was about 1 h. Addition of ANP or CNP  $10^{-8}$  to  $10^{-6}\text{ M}$  did not significantly affected any parameter of *S. aureus* MFP03 growth. The same observations were made using ANP and CNP ( $10^{-6}\text{ M}$  each) in association. At 33°C, the growth curves of *S. aureus* MFP03 were slightly different in comparison with ones at 37°C (**Figure 1B**). The growth constant was higher ( $0.63\text{ h}^{-1}$ ), and the generation time was slightly increased (63 min), but cells did not enter into a death phase characterized by reduction of OD, maybe because they grew slower (lag-phase 1.5 h) and with higher maximal OD<sub>580</sub> (about 0.97). As observed at 37°C, addition of NUPs did not significantly modified *S. aureus* growth parameters.

In contrast, we observed that the growth of *C. acnes* RT4 and RT5 at 37°C was impacted by NUPs exposure (**Figure 2**). More precisely, in control conditions the strain RT5 showed a growth constant of  $0.12\text{ h}^{-1}$ , a generation time of 7 h and a death rate constant at  $0.0003\text{ h}^{-1}$ . Addition of ANP and CNP markedly increased the generation time of this bacterium. The maximum reached 14.87 h in the presence of ANP  $10^{-8}\text{ M}$  (+ 112.4 %) and 11.29 h (+60.5 %) in the presence of CNP  $10^{-7}\text{ M}$ . However, association of the two NUPs only increased the generation time to 8.1 h (+15.7 %), and no additive or synergistic effect of ANP and CNP combination was observed. In presence of both ANP and CNP the growth constant varied from 0.05 to  $0.08\text{ h}^{-1}$ .

Concerning death rate constants, no variation was observed for all NUP concentration tested. For the strain RT4 in control condition, we measured a generation time of 7.8 h, a growth constant of  $0.09\text{ h}^{-1}$  and a death rate constant at  $0.015\text{ h}^{-1}$ . Addition of both peptides increased the generation time to 10.28 h (ANP  $10^{-6}\text{ M}$ ) or 12.25 h (CNP  $10^{-8}\text{ M}$ ) (**Figure. 2A**). When the bacteria were exposed to a combination of the two NUPs, no additional or synergistic effect was observed (the generation time remained of 8.1 h). The growth constant in the presence of NUPs slightly decreased and varied from

0.06 to 0.08 h<sup>-1</sup>. Probably because of this slight reduction of the growth rate, the death rate constant was lower and varied near zero. The RT4 strain maximum OD was increased in presence of CNP and slightly decreased in presence of ANP. The same was observed with the RT5 (**Figure 2B**) strain in the presence of CNP 10<sup>-6</sup> M. So, in the case of *C. acnes* it appeared that both NUPs were capable to modulate the growth dynamics, although these effects were generally moderate (Maximum OD<sub>580</sub> was varying between 0.8 and 1.4 for both strains, and in the case of the RT5 strain a marginal decrease was observed, except in the case of CNP 10<sup>-6</sup> M. At 33°C, the effects of both NUPs were virtually as marginal as observed at 37°C (*Data not shown*).

### **Confocal laser scanning microscopy of monospecies biofilms in presence of NUPs**

Analysis of the mean biofilm thickness and biofilm biomass density of *S. aureus* MFP03 revealed the crucial significance of cultivation conditions to observe ANP and CNP effects (**Figure 3**). It is interesting to note that the biggest impact was observed at low concentrations of both ANP and CNP, *i.e.* 10<sup>-8</sup> M. In the absence of treatment and in aerobic conditions at 37°C, the biomass density and the average thickness of the biofilm formed by *S. aureus* MFP03 were 27.66 ± 1.46 μm<sup>3</sup>/μm<sup>2</sup> and 30.13 ± 1.69 μm, respectively. At all tested concentrations, ANP inhibited *S. aureus* MFP03 biofilm growth. Addition of ANP 10<sup>-8</sup> M decreased the average biomass density of the biofilms to 18 ± 5.9 μm<sup>3</sup>/μm<sup>2</sup> (65.2 ± 21.5 % of the control, **Figure 3A**), and decreased the average thickness of the biofilms to 25.6 ± 1.9 μm (86.9 ± 6.4 % of the control, **Figure 3B**). CNP significantly inhibited *S. aureus* biofilms only at the lowest tested concentration (10<sup>-8</sup> M) where it decreased the biomass density to 17.7 ± 4 μm<sup>3</sup>/μm<sup>2</sup> (64.1 ± 14.35 % of the control) and the average biofilm thickness to 22 ± 0.5 μm (73.4 ± 1.7% of the control). Association of the two NUP (ANP and CNP; 10<sup>-6</sup> M each) had a slight synergistic inhibitory effect on the biofilm parameters: Individually ANP 10<sup>-6</sup> M decreased the average biomass density and thickness of the biofilms to 24.8 ± 0.9 μm<sup>3</sup>/μm<sup>2</sup> (89.8 ± 3.4 % of the control) and 27.1 ± 1 μm (90 ± 3.4 % of the control), respectively; CNP 10<sup>-6</sup> M alone reduced these values to 24.9 ± 3.2 μm<sup>3</sup>/μm<sup>2</sup> (90.1 ± 11.6 % of the control) and 25.8 ± 4.7 μm (85.5 ± 15.7 % of the control), respectively. Combination of two NUPs decreased the biomass density and thickness to 19.9



$\pm 0.7 \mu\text{m}^3/\mu\text{m}^2$  ( $72.1 \pm 2.6$  % of the control) and to  $22.11 \pm 0.5 \mu\text{m}$  ( $73.4 \pm 1.7\%$  of the control), respectively. At  $33^\circ\text{C}$  in aerobic conditions no effect of the peptides was observed, except in terms of mean thickness where an increase was observed in the presence of CNP  $10^{-8}$  M. More precisely, at  $33^\circ\text{C}$  in aerobic conditions the average biomass density was only of  $14.07 \pm 0.85 \mu\text{m}^3/\mu\text{m}^2$ , and the average biofilm thickness was falling to  $14.1 \pm 0.9 \mu\text{m}$ . Then, at  $33^\circ\text{C}$  and in aerobic conditions *S. aureus* MFP03 showed a reduced biofilm formation activity in comparison to that observed aerobically at  $37^\circ\text{C}$ . When ANP or CNP were added, the biofilm biomass density varied from  $14.21 \pm 1 \mu\text{m}^3/\mu\text{m}^2$  ( $101 \pm 7\%$  of the control) to  $15.9 \pm 0.84 \mu\text{m}^3/\mu\text{m}^2$  ( $113 \pm 6\%$  of the control after exposure to CNP  $10^{-8}$  M). The average thickness was varying from  $13.74 \pm 1.3 \mu\text{m}$  ( $97.5 \pm 9\%$  of the control) to  $16.3 \pm 1.3 \mu\text{m}$  ( $116 \pm 9\%$  of the control with CNP  $10^{-8}$  M). Then, at  $33^\circ\text{C}$  the weak inhibitory effect of both NUPs observed at  $37^\circ\text{C}$  disappeared and a tendency to stimulation emerged.

When *S. aureus* was grown in anaerobic conditions at  $33^\circ\text{C}$ , we observed that the biofilms reached a biomass density of  $16.7 \pm 0.58 \mu\text{m}^3/\mu\text{m}^2$  and an average thickness of  $17.3 \pm 0.66 \mu\text{m}$ . These biofilms appeared more dense and thick than at  $33^\circ\text{C}$  in aerobic atmosphere, but remained less developed than at  $37^\circ\text{C}$ . High concentrations ( $10^{-6}$  M) of both peptides did not significantly affected biofilms formation in these growth conditions (anaerobic,  $33^\circ\text{C}$ ). In contrast, we observed that peptides used at  $10^{-7}$  M and  $10^{-8}$  M stimulated biofilm growth (**Figure 3**). In the presence of ANP  $10^{-8}$  M, the biofilm biomass density reached  $22.8 \pm 3.1 \mu\text{m}^3/\mu\text{m}^2$  ( $136.6 \pm 19\%$  of the control) and an average thickness of  $23.2 \pm 3.6 \mu\text{m}$  ( $134.9 \pm 21$  % of the control). In the same conditions, CNP increased the average biomass density and thickness up to  $21.1 \pm 1.7 \mu\text{m}^3/\mu\text{m}^2$  ( $126.8 \pm 10\%$  of the control) and  $21.2 \pm 1.7 \mu\text{m}$  ( $122.7 \pm 10\%$  of the control), respectively. At a concentration of  $10^{-7}$  M the effect of CNP on the biofilm density and thickness was more limited ( $112 \pm 13$  % and ( $111 \pm 13$  % of the control, respectively). Exposure of *S. aureus* to both peptides in association didn't bring any advantage and didn't have any additional effects. Moreover, changing TSB medium to RCM was leading to the disappearance of all significant stimulatory effects of NUPs (**Figure 3**).



When the same experiment was realized on *C. acnes*, we observed that both strains were sensitive to NUPs. Of course, as RT4 and RT5 strains are unable to grown in the presence of oxygen, the effect of NUPs was only investigated in anaerobic conditions. At 37°C the biofilm formation activity of *C. acnes* RT4 was inhibited by both NUPs alone or in association (**Figures 4A and 4C**). In control conditions the biomass density and average thickness of the biofilm formed by *C. acnes* RT4 were  $20.1 \pm 0.4 \mu\text{m}^3/\mu\text{m}^2$  and  $23.2 \pm 0.5 \mu\text{m}$ , respectively. ANP maximally reduced RT4 biofilms when  $10^{-7}$  M: density and thickness decreased to  $12.5 \pm 2.6 \mu\text{m}^3/\mu\text{m}^2$  ( $62.3 \pm 13\%$  of the control) and  $15.9 \pm 2.5 \mu\text{m}$  ( $68.5 \pm 11\%$  of the control), respectively. When ANP was used at a concentration of  $10^{-8}$  M we observed the same effect. CNP was more efficient at a concentration of  $10^{-7}$  M with thickness and density biofilms values that reached only  $51 \pm 12\%$  and  $49 \pm 13\%$  of the control, respectively. Combination of ANP and CNP had a limited additive effect on *C. acnes* RT4 biofilm formation. NUPs also exerted an inhibitory effect on the biofilm formation activity of *C. acnes* RT5 when it was grown at 37°C. In control conditions at 37°C *C. acnes* RT5 has a strong biofilm formation activity ( $26.9 \mu\text{m}$  average thickness and  $23.3 \mu\text{m}^3/\mu\text{m}^2$  biomass density) (**Figures 4B and 4D**). At all tested concentrations ANP inhibited *C. acnes* RT5 biofilm density and thickness with values that reached 67 to 79 % and 75.5 to 79.5 % of the control). The maximal effect of CNP was noted at  $10^{-6}$  M with a decrease of the biofilm thickness and density that reached  $42.3 \pm 17$  and  $49.2 \pm 16\%$  of the control, respectively. CNP  $10^{-7}$  M had virtually the same effect. In contrast, combination of ANP and CNP did not result in any an additive or a synergistic effect.

When the incubation temperature of the two *C. acnes* strains was reduced to 33°C, their biofilm formation activity and sensitivity to ANP and CNP was different. The RT4 strain produced thinner biofilms of  $8.9 \pm 0.49 \mu\text{m}^3/\mu\text{m}^2$  biomass density and  $9.3 \pm 0.5 \mu\text{m}$  thickness. Moreover, no inhibitory effect of ANP and CNP was observed and even a marginal stimulation of the biomass density was noted (**Figures 4A and 4C**), especially in presence of ANP. Indeed, ANP  $10^{-7}$  M increased RT4 strain biofilm biomass density and average thickness to  $121.1 \pm 24\%$  and  $108.3 \pm 19\%$  of the control. The biofilm formation activity of *C. acnes* RT5 at 33°C was also decreased but the effect was more

marginal than on the RT4 strain with an average thickness and biomass density of  $20.7 \pm 0.9 \mu\text{m}^3/\mu\text{m}^2$  and  $21 \pm 1 \mu\text{m}$ , respectively (**Figures 4 B and 4D**). The inhibitory effect of ANP and CNP was preserved although it was weaker. The stronger effect of ANP was observed at a concentration of  $10^{-8}$  M with a decrease of biofilms density and thickness to  $77 \pm 12$  and  $77 \pm 13$  % of the control, respectively. CNP exerted a strongest inhibitory effect when it was used at a concentration of  $10^{-7}$  M leading to a. More precisely, biofilms thickness and biomass density of  $68.5 \pm 11$  and  $67 \pm 1$  % of the control, respectively. This is showing that the effect of NUPs on *C. acnes* is generally inhibitory but is also strain-specific and can be modulated by the microenvironment. This should be correlated to the behavior and ecological role of *C. acnes* strains in the different human skin niches. On another side, the general lack of additive or synergistic effect of ANP and CNP suggests that the two peptides should compete for their action on the bacterium.

#### **Interaction of *S. aureus* MFP03 and *C. acnes* in mixed biofilms**

First, we analyzed how the co-existence of *S. aureus* MFP03 and *C. acnes* strains in mixed biofilms produced on glass fiber filters can affect their growth at 37°C. We rapidly observed that in biofilms *C. acnes* forms very solid microcolonies and that these aggregates are impossible to disperse as single cells even using sonication (*Data not shown*). Therefore we decided to measure cultivable bacteria present in the biofilm by direct counting of *S. aureus* CFU from dispersed biofilms and to determine the metabolic activity present in the biofilms by MTT staining allowing to estimate indirectly the evolution of *C. acnes* biomass.

An average amount of  $3.2 \times 10^7 \pm 4.1 \times 10^6$  *S. aureus* CFU was distributed on the filters. After 72h of culture at 37°C in monospecies biofilms the number of cultivable *S. aureus* MFP03 recovered from the monospecies biofilms reached a mean of  $6.1 \times 10^8 \pm 1.2 \times 10^8$  CFU. In the presence of *C. acnes*, *S. aureus* grew better. More precisely, using the *C. acnes* RT4 strain or the *C. acnes* RT5 strain ( $8.75 \times 10^9 \pm 3.9 \times 10^9$  CFU) in co-cultures the amount of *S. aureus* MFP03 CFU recovered in the presence of the RT4 and RT5 strains of *C. acnes* reached  $2.62 \times 10^9 \pm 1.3 \times 10^9$  CFU and  $8.75 \times 10^9 \pm 3.9 \times 10^9$ , respectively (**Figure 5A**). The MTT staining of biofilms shown that the average staining

intensity of monospecies *S. aureus* MFP03 and *C. acnes* biofilms was virtually equal: OD<sub>595</sub> was 1.17 ± 0.13 for *S. aureus*, 1.28 ± 0.21 for *C. acnes* RT4, and 1.21 ± 0.22 for *C. acnes* RT5, respectively (**Figure 5B**). However, it is interesting to note that in the case of mixed biofilms, the MTT values are far to be equal to the sum of monospecies biofilms values. As in addition the CFU counts show that *S. aureus* was growing better in mixed biofilms, these results suggest that the growth of *C. acnes* was reduced in the presence of *S. aureus* MFP03.

To simulate better the skin microenvironment where *C. acnes* and *S. aureus* coexist, we decided to study mixed biofilms at 33°C as the skin surface is not at 37°C and much more close to 33°C (Ariyaratnam & Rood, 1990; Boutcher et al., 1995). Also, to analyze and model probable effects of colonization, we studied different types of mixed biofilms. We used only strain RT5 because in experiments with CLSM of monospecies biofilms it demonstrated inhibitory effect when it was exposed to ANP and CNP at 33°C, whereas *S. aureus* MFP03 was insensitive to NUPs at this temperature. We observed that in case of simultaneously grown mixed biofilms and in mixed biofilms for which pre-formed *S. aureus* MFP03 biofilm was first formed, there was no significant difference between CFU amount of *S. aureus* MFP03 in monospecies and mixed biofilms (**Figure 6A**). In the case of mixed biofilms based on pre-formed *C. acnes* biofilms, the amount of *S. aureus* MFP03 CFU recovered from monospecies biofilms was higher than from mixed biofilm ( $7.6 \times 10^9 \pm 3.8 \times 10^8$  and  $4.6 \times 10^9 \pm 3 \times 10^8$  CFU, respectively). Likewise, in the case of *C. acnes*-based biofilms, the amount of *S. aureus* CFU in monospecies biofilms was higher than in other cases studied:  $7.6 \times 10^9 \pm 3.8 \times 10^8$  against  $4.8 \times 10^9 \pm 1.3 \times 10^9$ ,  $4.5 \times 10^9 \pm 7.2 \times 10^8$  and  $4.2 \times 10^9 \pm 6.8 \times 10^8$  CFU for simultaneously grown biofilms, aerobically grown *S. aureus*-based biofilms and anaerobically grown *S. aureus* biofilms, respectively. That should be explained by cell death level in long-term incubated monospecies *S. aureus* MFP03 biofilms. Indeed, in case of simultaneously grown biofilms both monospecies and mixed biofilms were incubated for 3 days whereas in the case of pre-formed biofilms *S. aureus* MFP03 biofilms needed to be grown for 4 days (with replacing of filters with biofilms on fresh medium).

The MTT staining of monospecies and mixed biofilms at 33°C (**Figure 6B**) shown that for each type of mixed biofilm, the MTT staining value was less than sum of the intensities obtained independently for each monospecies biofilms, suggesting that *C. acnes* RT5 was less growing in mixed biofilms than in monospecies ones. Nevertheless, this inhibitory effect was not as marked as in studies realized at 37°C, especially in the case of pre-formed *C. acnes* RT5 biofilms. More precisely, the OD<sub>595</sub> of formazan extracts in that case were  $1.92 \pm 0.04$  and  $1.7 \pm 0.21$  for monospecies *S. aureus* MFP03 and *C. acnes* RT5 biofilms, respectively, but reached  $2.955 \pm 0.035$  for the binary biofilms, *i.e.* 81.6 % of the algebraic sum of the two monospecies biofilms (**Figure 6B**). These data suggest that *C. acnes* in mature biofilm can tolerate negative effects of *S. aureus* MFP03, and that mature biofilms of *C. acnes* are not favorable for *S. aureus* MFP03 adhesion.

#### **Effects of ANP and CNP on mixed biofilms formed by *C. acnes* RT5 and *S. aureus* MFP03**

To investigate the potential effects of NUPs on mixed biofilms we used liquid RCM and grew biofilms simultaneously in equilibrium with planktonic cultures. We decided to test concentrations of NUPs which had the strongest effect on monospecies biofilms in experiments with CLSM, *i.e.*  $10^{-8}$  M for ANP and  $10^{-7}$  M for CNP.

In presence of NUPs, the number of *S. aureus* MFP03 CFU obtained from monospecies biofilms was decreased (**Figure 7A**) ranging from  $1.76 \times 10^9 \pm 1.1 \times 10^8$  CFU in control studies to  $1.64 \times 10^9 \pm 2 \times 10^8$  CFU with ANP and only  $7.58 \times 10^8 \pm 2.2 \times 10^8$  CFU with CNP. This suggests that, if cells have to adhere to surface from a planktonic state in the presence of the peptides, NUPs should interfere with this process. This can be an explanation of the decrease of cultivable bacteria obtained from experiments realized in static conditions on filters since biofilm production for CLSM experiments cells requires an initial adhesion step.

In mixed biofilms, the number of *S. aureus* MFP03 CFU measured was 2.15 fold less than in monospecies biofilms ( $8.1 \times 10^8 \pm 3 \times 10^8$  CFU, **Figure 7A**). Addition of ANP increased this reduction of *S. aureus* development in mixed biofilms (3.3-fold reduction with an average of  $5 \times 10^8 \pm 4 \times 10^7$  CFU recovered from the biofilms). In contrast, addition of CNP did not change significantly the ratio

between planktonic and biofilm bacteria in monospecies and binary biofilms although in binary biofilms the number of *S. aureus* MFP03 was 2.33-fold lower than in mixed ones ( $3.25 \times 10^8 \pm 4.1 \times 10^7$  CFU).

It was interesting to note that *S. aureus* CFU count results were correlated to MTT values (**Figure 7B**). MTT staining, corresponding to the metabolic activity in biofilms, was reduced in mixed biofilm in comparison to *S. aureus* MFP03 monospecies biofilms grown in the presence of ANP and CNP (-17.4 % and -25.4 %). The same was observed with *C. acnes* RT5 where a reduction of the metabolic activity of 18.7 % was observed between monospecies and binary biofilms in the presence of ANP or CNP. However, the more striking result was that the MTT value of mixed biofilms was of the same range in control studies ( $OD = 0.65 \pm 0.08$ ), and in the presence of ANP ( $0.61 \pm 0.1$ ) or CNP ( $0.72 \pm 0.13$ ). These data suggest that *S. aureus* MFP03 was the dominant component of dual-species biofilm but more importantly, considering the absence of variation of MTT values in binary biofilms and the decrease of cultivable *S. aureus* MFP03, that the balance was shifted to *C. acnes* RT5 in presence of NUPs.

Considering these results and hypothesis, we decided to go further by investigating by confocal microscopy mixed biofilms labeled with a FISH probe for *C. acnes*. In control studies, we exposed monospecies biofilms of *S. aureus* with this probe for *C. acnes* and we observed no false hybridization. Then, we calculated the percentage of hybridization for each repeat in mixed biofilms and in parallel we stained with the FISH-probe monospecies *C. acnes* RT5 biofilms (*Data not shown*). Coefficient varied from 0.2 to 0.99. DAPI was used as an indicator of total biomass in binary biofilms. These values were used to calculate *C. acnes* biomass in mixed biofilms. NUPs were tested at the same concentration as described above.

Using simultaneously formed biofilms, in control studies the DAPI labelling illustrating the total bacterial was  $2.93 \pm 1.2 \mu\text{m}^3/\mu\text{m}^2$ . In presence of ANP and CNP it was of  $2.87 \pm 1.6$  and  $3.06 \pm 1.1 \mu\text{m}^3/\mu\text{m}^2$  respectively, showing no changes in totally biomass density (**Figure 8**). However, in all types of mixed biofilms, addition of ANP or CNP increased *C. acnes* RT5 biomass. Indeed, the biomass of

*C. acnes* RT5 passed from  $0.36 \pm 0.18 \mu\text{m}^3/\mu\text{m}^2$  in control studies to  $0.85 \pm 0.53$  and  $0.54 \pm 0.28 \mu\text{m}^3/\mu\text{m}^2$  in the presence of ANP and CNP, *i.e.* +236 and +150 % respectively. Taking in reference *C. acnes* biomass in monospecies biofilms, these results demonstrate that the proportion of *C. acnes* RT5 in binary biofilms in the presence of ANP and CNP increased to 31 and 18%, respectively whereas it was only of 12% in control biofilms 12% (**Figures 8 A-C, Figure 9A**). These results are coherent with CFU counts and MTT measurements.

As noted in simultaneously formed biofilms, using binary biofilms grown on the basis of *S. aureus* MFP03 pre-formed biofilms we observed that ANP increased *C. acnes* RT5 biomass. In control studies it was of  $2.9 \pm 1.1 \mu\text{m}^3/\mu\text{m}^2$  (**Figure 8D**) and reached  $3.1 \pm 1.6 \mu\text{m}^3/\mu\text{m}^2$  after exposure to ANP (**Figure 8E**). Conversely, the biomass of *C. acnes* measured after exposure to CNP decreased to  $0.8 \pm 0.4 \mu\text{m}^3/\mu\text{m}^2$  (**Figure 8F**). This huge reduction of *C. acnes* biomass in the presence of CNP can be correlated to the strong reduction of *S. aureus* MFP03 CFU count previously observed, suggesting that in these conditions CNP should exert a strong inhibitory effect on biofilm formation. However, it is interesting to note that the calculated relative percentage of *C. acnes* produced on pre-formed *S. aureus* biofilms evolved from 21 % in control studies to 40.3 and 36.3 % in the presence of ANP and CNP (**Figure 9B**) suggesting that *S. aureus* biofilm is favorable for *C. acnes* adhesion and that the decrease in *C. acnes* biomass in biofilms observed with CNP should result from the strong reduction of *S. aureus* development provoked by this NUP.

This hypothesis is coherent with results from studies realized on mixed biofilms based on pre-formed *C. acnes* RT5 biofilms showing and increase in total biofilm biomass from  $2.7 \pm 1.3$  to  $2.9 \pm 0.5$  and  $3.4 \pm 0.7 \mu\text{m}^3/\mu\text{m}^2$  under the effect of ANP and CNP, respectively. This increase can be essentially attributed to a rise of the specific *C. acnes* RT5 biomass which evolved from 55% in control studies to 68.2% and 74.5% after exposure to ANP and CNP, respectively (**Figures 8G-I and 9C**). Although, particularly in the case of *S. aureus*, the differences of growth kinetics of the two bacterial species could impact their survival in studies requiring long-term cultivation, these results suggest that the surface of *C. acnes* biofilms should not be favorable to *S. aureus* adhesion and development.

**Screening for putative sensor for ANP and CNP in *S. aureus* and *C. acnes* by protein-BLAST**

Although identification of NUPs sensor(s) in *S. aureus* and *C. acnes* should represent another total complete study, in order to understand the effect of ANP and CNP we investigated by protein BLAST the presence in these bacteria of proteins showing homology with the amidase AmiC. This protein is showing high structural homology with the human C-Type NUP receptor and was identified as the CNP sensor in *P. aeruginosa* (Rosay et al., 2015). By analysis using protein sequences of collection strains (NCBI database) we found that the N-acetomuramoyl-L-alanilamidase of *C. acnes* (NCBI reference sequence WP\_042852295.1) is presenting 69% homology with the *P. aeruginosa* PAO1 AmiC. No significant homology with the human NUPs receptor was found in the sequenced genome of *S. aureus* but we noted that another amidase of Propionibacteriaceae (NCBI reference sequence WP\_002530699.1) is showing 50% homology with the GatA subunit of the aspartate-tRNA/glutamate-tRNA-amidotransferase of *S. aureus* (NCBI reference sequence WP\_072460603.1). These enzymes catalyze the same reaction of N-C bond cleavage, but in different cell processes: AmiC in *P. aeruginosa* is involved in the regulation of the amidase operon expression (Wilson & Drew, 1991), the *C. acnes* amidase is involved in cell cycle and cell wall development, and the *S. aureus* amidotransferase is indirectly involved in amino acid exchange and protein synthesis. These bioinformatics studies support the hypothesis, as *P. aeruginosa*, *S. aureus* and *C. acnes* could express AmiC related proteins with could act as NUPs sensors and provide a potential support for these first physiological observations.



**DISCUSSION**

Natriuretic peptides which are synthesized and released by cardiomyocytes (ANP) and endothelium cells of capillary vessels (CNP), reach different zones of the organism through bloodstream and local diffusion where temperature and oxygen concentrations are quite different. Skin is one of these organs characterized by important local variations of the microenvironment. Indeed, whereas in surface and at the level of the stratum corneum the percentage of oxygen is that of the atmosphere (21%) it falls to 3% at the basis of the stratum spinosum (Grillon et al., 2012), leading to microaerophile and even probably anaerobic conditions in specific niches. Similarly, the abundance of anastomoses in skin is allowing important changes of the microcirculation and therefore of the local temperature. In particular, in forehead skin, a site where acne appears very frequently, the mean temperature is close to 33°C (Ariyaratnam & Rood, 1990; Boucher et al., 1995). The hair follicle and sebaceous gland, from which acne generally emerges, is then exposed to very specific environmental conditions in addition to high local concentrations of neuroendocrine factors such as NUPs that can be released by the dense capillary network present at the level of the bulge (Xiao et al., 2013). Because of the very small distance between capillary vessels and skin microbial community in that zone, lipophilic bacteria such as *C. acnes* and *S. aureus* are therefore exposed in permanence to NUPs.

*C. acnes* is one of predominant components of the skin microbiome (Pettersson et al., 2018). It is suspected to be involved in acne formation but its role remains unclear (Tomida et al., 2013). Indeed, inside skin glands and hair follicles hundreds of microbial species live, thus, it is premature to designate *C. acnes* as the only causative agent of acne. Recent studies have shown that *S. aureus* can be opulent inside inflammatory acne ulcers (Totté et al., 2016; Dreno et al., 2017). To date there are no evidences that *S. aureus* can be a causative agent of acne vulgaris, but its role in acne formation is expected. In addition we can mention that *S. aureus* colonizes plenty skin areas affected by psoriasis and atopic dermatitis (Elfatoiki et al., 2016; Lacey et al., 2016), and causes inflammation and treatment complications. Inside skin glands and hair follicles quite aerotolerant *C. acnes* prefer to colonize



570 anaerobic micro-niches (Matard et al., 2013), where they forms complex biofilms which include *S.*  
571 *aureus*.

572 We have recently shown that NUPs can affect significantly *Staphylococcus epidermidis* and *S.*  
573 *aureus* biofilms formation activity (Gannesen et al., 2018). Now we shown the effect of ANP and CNP  
574 in the regulation of monospecies and mixed biofilms of *C. acnes* and *S. aureus* and explored for the  
575 first time the influence of oxygen concentrations and temperature. It appears that NUPs are involved  
576 deeply in the interactions between the human skin and its microbiome. NUPs affect mostly biofilms  
577 and less planktonic cultures, that could be explained by the fact that in the human organism microbes  
578 live predominantly in multispecies biofilms. This is particularly specific for skin where physico-  
579 chemical conditions are not allowing the bacteria to grown in planktonic form. We demonstrate that  
580 NUPs have not only lots of regulatory functions in human organism as hormones, but also that NUPs  
581 are able to affect the commensal skin human microbiota. It broadens horizons of our understanding of  
582 how human hormone system works. Besides that, with this data we became closer to understanding of  
583 global interactions between commensal microbiota and human organism. NUPs interaction with skin  
584 microbiota and their regulatory functions can be a result of thousands years of co-evolution of humans  
585 and their microbiome. It can be approved also by the fact that we found a high homology of AmiC the  
586 CNP receptor in *P. aeruginosa* (Rosay et al., 2015) and *C. acnes* amidase and *S. aureus*  
587 amidotransferase.

588 Differences in effects and their dependence on concentration for different microorganisms can be  
589 an evolutionary adaptation of human hormone system for interaction with microbiota. At first view  
590 illogical effects, independent of NUPs concentrations were observed, particularly in the case of *S.*  
591 *aureus* MFP03. However, NUPs are known to form aggregates at high concentrations (Torricelli et al.,  
592 2004) that could limit their activity and make it less efficient than at low doses. In addition, *S. aureus*  
593 mainly prefers to colonize the surface of moist skin areas like nostrils (Estes, 2011; Feuerstein et al.,  
594 2017). Then, *S. aureus* should be generally more remote from capillaries than *C. acnes* and therefore

inhabits in areas where the NUPs concentrations should be lower and by this way more efficient on this bacterial species.

When we investigated *C. acnes* monospecies biofilms we observed that the action of ANP and CNP was strain specific suggesting that, as proposed for *S. aureus*, the sensitivity of the different *C. acnes* strains to NUPs should be related to their original microenvironment in skin. Whereas *C. acnes* RT5 was sensible to NUPs at 37 and 33 °C, *C. acnes* RT4 became non sensitive at 33°C. This could reflect different roles and involvements in acne formation of the *C. acnes* strains and then differences in human skin adaptation although both RT4 and RT5 were considered as acneic. We focused our work on the *C. acnes* RT5 strain because in monospecies biofilms it showed a biofilm formation inhibitory response to ANP and CNP at 33°C suggesting a complex adaptation to the human skin physiology and longtime close interactions between this strain and the human hormone systems.

Then we constructed binary systems where *S. aureus* MFP03 and *C. acnes* RT5 were sensible to NUPs to analyze the interrelations of the two bacteria in a same biofilm and the potential interference with NUPs. First we studied the growth of bacteria on glass fiber filters on Petri dishes then in the absence equilibrium with planktonic culture in a liquid compartment. In these conditions, binary systems of *C. acnes* RT5 and *S. aureus* MFP03 grew better when the two bacteria were cultivated together than in monospecies biofilms. However, analysis of MTT values showed that the metabolic activity of mixed biofilms was lower than the sum of monospecies biofilms. This should result from the lower growth kinetics of *C. acnes* in comparison to *S. aureus* in mixed biofilms making *C. acnes* a weak competitor in front of *S. aureus*. However, when *C. acnes* RT5 was allowed to form biofilm before interaction with *S. aureus*, *C. acnes* RT5 resisted very well to *S. aureus*, and the growth of *S. aureus* was partly impaired. Nevertheless, generally the presence of *C. acnes* RT5 affected neutrally or positively the growth of *S. aureus* MFP03.

The effect of NUPs on binary biofilms was studied in another system where biofilms were grown in equilibrium with planktonic cells. This model was selected because it was simpler to work with NUPs and because of the high cost of the peptides that required to use limited volumes. In this system

the growth of *S. aureus* MFP03 was reduced in the presence of *C. acnes* RT5 in comparison to monospecies biofilms despite its higher growth rate. This is revealing the dependence of adhesion and the importance of interactions between microorganisms on the very early biofilm formation steps. When the cells were forced to adhere on filters on Petri dishes, *S. aureus* MFP03 had great advantage because of its growth speed. Conversely, when *S. aureus* had to adhere to fibers in the presence of *C. acnes* its growth was reduced, indicating that *C. acnes* RT5 was able to interrupt the adhesion of *S. aureus* MFP03 to glass fibers. Monospecies biofilms of both microorganisms were inhibited in this system in presence of both NUPs, but in binary biofilm *C. acnes* RT5 grew better in presence of both NUPs, suggesting that NUPs could increase the competitive advantages of *C. acnes* RT5. Considering that at 33°C *C. acnes* RT4 became non-sensitive to NUPs, these data suggest that NUPs can exert a highly specific control on some strains of *C. acnes*, increasing or not their competitive advantages in comparison to their neighbors in the complex microbial community of hair follicles and skin glands.

These results are leading to propose a functional hypothesis concerning the potential role of NUPs in the skin microbiota. Hence, in the forehead skin, where *C. acnes* and *S. aureus* are present in abundance, in normal healthy conditions the temperature is about 33°C and NUPs increase the competitive advantages of *C. acnes* while they inhibit the growth of *S. aureus* which is considered more pathogenic and potentially troublesome than *C. acnes* (Brown et al., 2013). In circumstances favorable for *C. acnes* overgrowth (skin pores plugging, comedone formation...), induction of the inflammatory reaction increases the local temperature to 37°C (Casscells et al., 1996; Changmugam et al., 2017). This increase of temperature should stimulate an intensive growth of *S. aureus* and in a lower extent that of *C. acnes* with a risk of runaway phenomenon. However, at 37°C NUPs should start to inhibit the growth of both bacteria and thus act as a thermostat to help human defense systems to reduce inflammation. By the same way, this temperature dependent effect ANP and CNP should be involved in pathologies such as psoriasis characterized by wavelike progress (Ayala-Fontáñez et al., 2016) and *S. aureus* overgrowth (Totté et al., 2016). Indeed, it appears that in psoriatic plaques, *S. aureus* have strong advantages against other microbial species because of unknown reasons, and

because of this it probably forms virtually monospecies biofilms. In remission phases, when the temperature of the skin is about 33°C, *S. aureus* should slowly form biofilms which can cause inflammation and a local temperature increase. While inflammation progresses, with the rise of the temperature NUPs should start to inhibit *S. aureus* biofilms formation. This hypothesis is supported by the fact that most effective concentrations of ANP and CNP active on *S. aureus* are low and *S. aureus* colonizes sites where the NUPs concentration should be low. However, during psoriasis progress an exuberant angiogenesis occurs, and by this way concentrations of NUPs should increase and their inhibitory effect on *S. aureus* vanish. That could explain at least by part because *S. aureus* is so difficult to eradicate on psoriatic lesions. Of course these hypotheses remain speculative and deserve further clinical investigations.

In conclusion, more than providing a further demonstration of the diversity and complexity of skin-bacteria communication, this work provides for the first time a potential functional role of ANP and CNP as natural thermostats allowing the organism to regulate bacterial biofilm development and balance between *C. acnes* and *S. aureus* as a function of the local temperature.

### ACKNOWLEDGEMENTS

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**LEGENDS TO FIGURES**

**Figure 1.** Growth dynamics of *S. aureus* MFP03 monospecies cultures in the presence of ANP and CNP at different temperatures A - 37°C; B – 33°C.

**Figure 2.** Growth dynamics of *C. acnes* RT4 and RT5 monospecies cultures at 37°C in the presence of ANP and CNP. A – *C. acnes* RT4; B – *C. acnes* RT5

**Figure 3.** Parameters of monospecies biofilms of *S. aureus* MFP03 measured by CLSM in different cultivation conditions. A – Average biofilms biomass density; B – Average biofilms thickness.

**Figure 4.** Parameters of monospecies biofilms of *C. acnes* measured by CLSM in different cultivation conditions. A – Average *C. acnes* RT4 biofilms biomass density; B – Average *C. acnes* RT5 biofilms biomass density; C – Average *C. acnes* RT4 biofilms thickness; D – Average *C. acnes* RT5 biofilms thickness.

**Figure 5.** Parameters of mixed biofilms of *S. aureus* MFP03 and *C. acnes* strains at 37°C. A – *S. aureus* MFP03 CFU; B – MTT biofilms values.

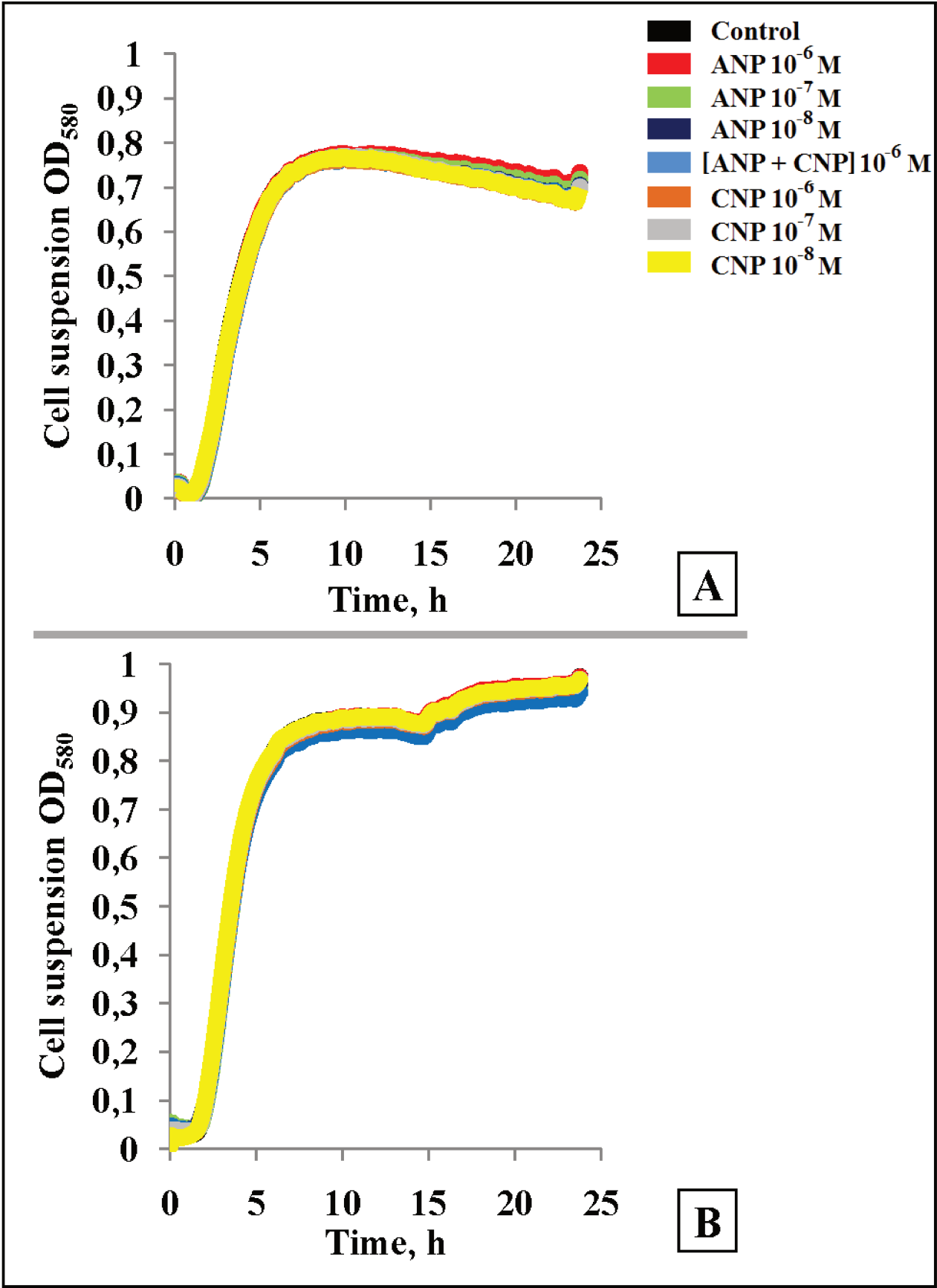
**Figure 6.** Parameters of different type mixed biofilms of *S. aureus* MFP03 and *C. acnes* RT5 at 33°C. A – *S. aureus* MFP03 CFU; B – MTT biofilms values.

**Figure 7.** Effects of ANP and CNP on mixed biofilms of *S. aureus* MFP03 and *C. acnes* RT5 at 33°C. A – *S. aureus* MFP03 CFU; B – MTT biofilms values.

**Figure 8.** CLSM images of FISH-labeled mixed biofilms of *S. aureus* MFP03 and *C. acnes* RT5. A – Simultaneously grown biofilm, Control; B – Simultaneously grown biofilm, ANP  $10^{-8}$  M; C - Simultaneously grown biofilm, CNP  $10^{-7}$  M; D – Biofilm based on pre-formed *S. aureus* MFP03 biofilm, Control; E – Biofilm based on pre-formed *S. aureus* MFP03 biofilm, ANP  $10^{-8}$  M; F - Biofilm based on pre-formed *S. aureus* MFP03 biofilm, CNP  $10^{-7}$  M; G – Biofilm based on pre-formed *C. acnes* RT5 biofilm, Control; H – Biofilm based on pre-formed *C. acnes* RT5 biofilm, ANP  $10^{-8}$  M; I - Biofilm based on pre-formed *C. acnes* RT5 biofilm, CNP  $10^{-7}$  M. Pink = *C. acnes* RT5 FISH labeling, Blue = DAPI staining (Total biomass).

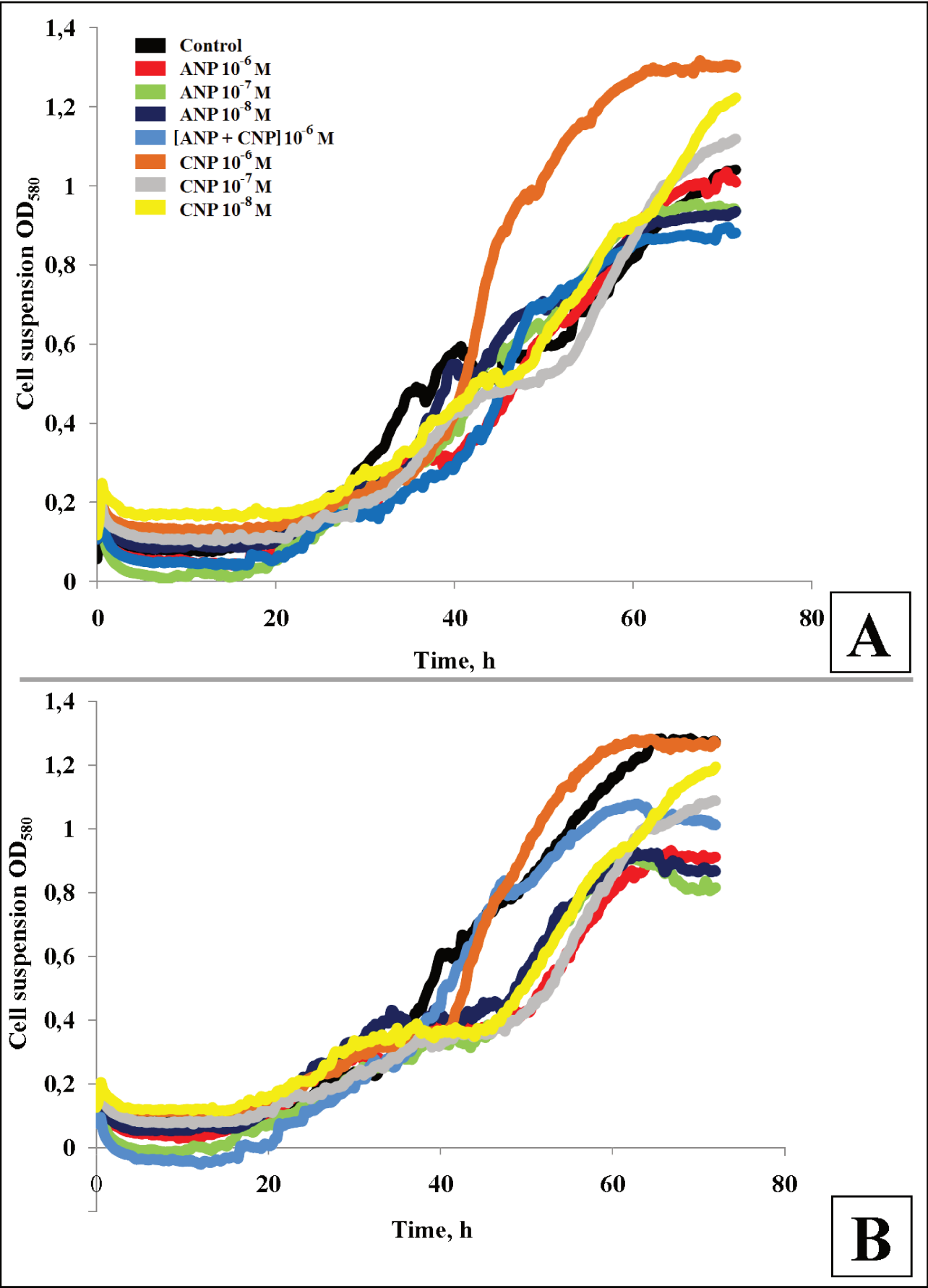
**Figure 9.** Contribution of *C. acnes* RT5 and *S. aureus* MFP03 to binary biofilms formed in the absence or presence of ANP or CNP. A - Simultaneously grown biofilms; B - Biofilms based on pre-formed *S. aureus* MFP03 biofilms; C - Biofilms, based on pre-formed *C. acnes* RT5.

878 **FIGURES**



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880 Figure 1.



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882 Figure 2.

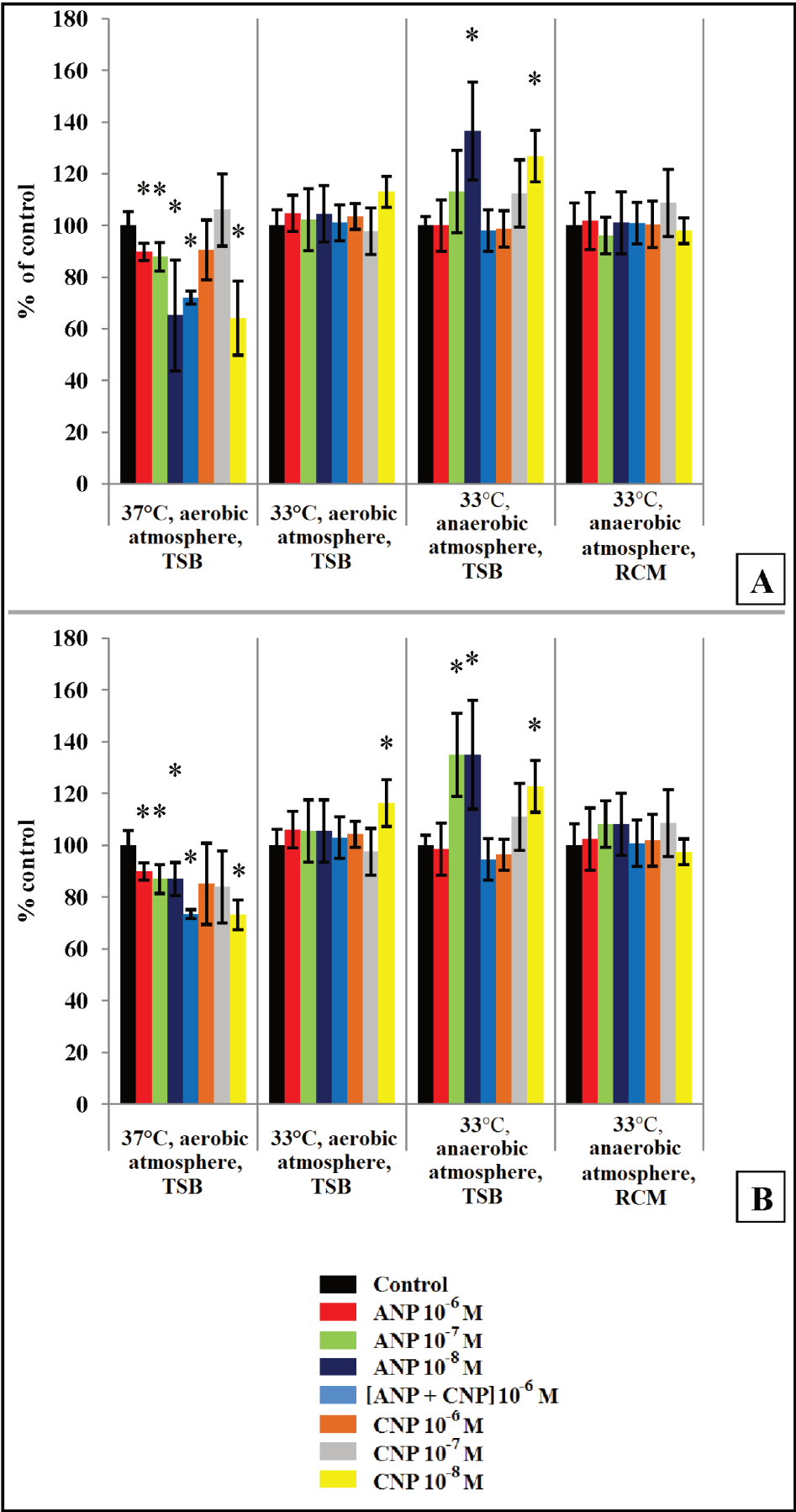


Figure 3.

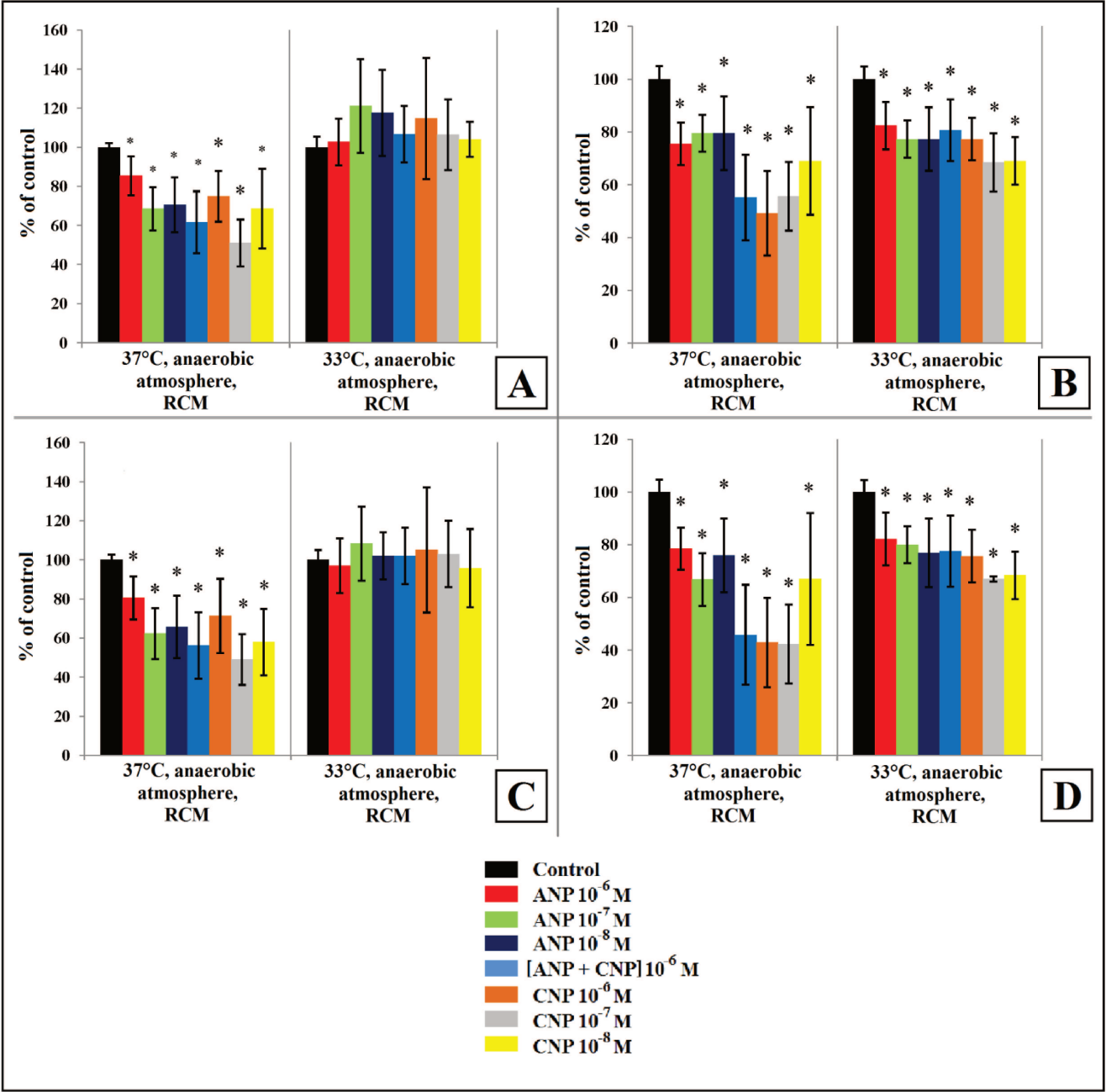


Figure 4.



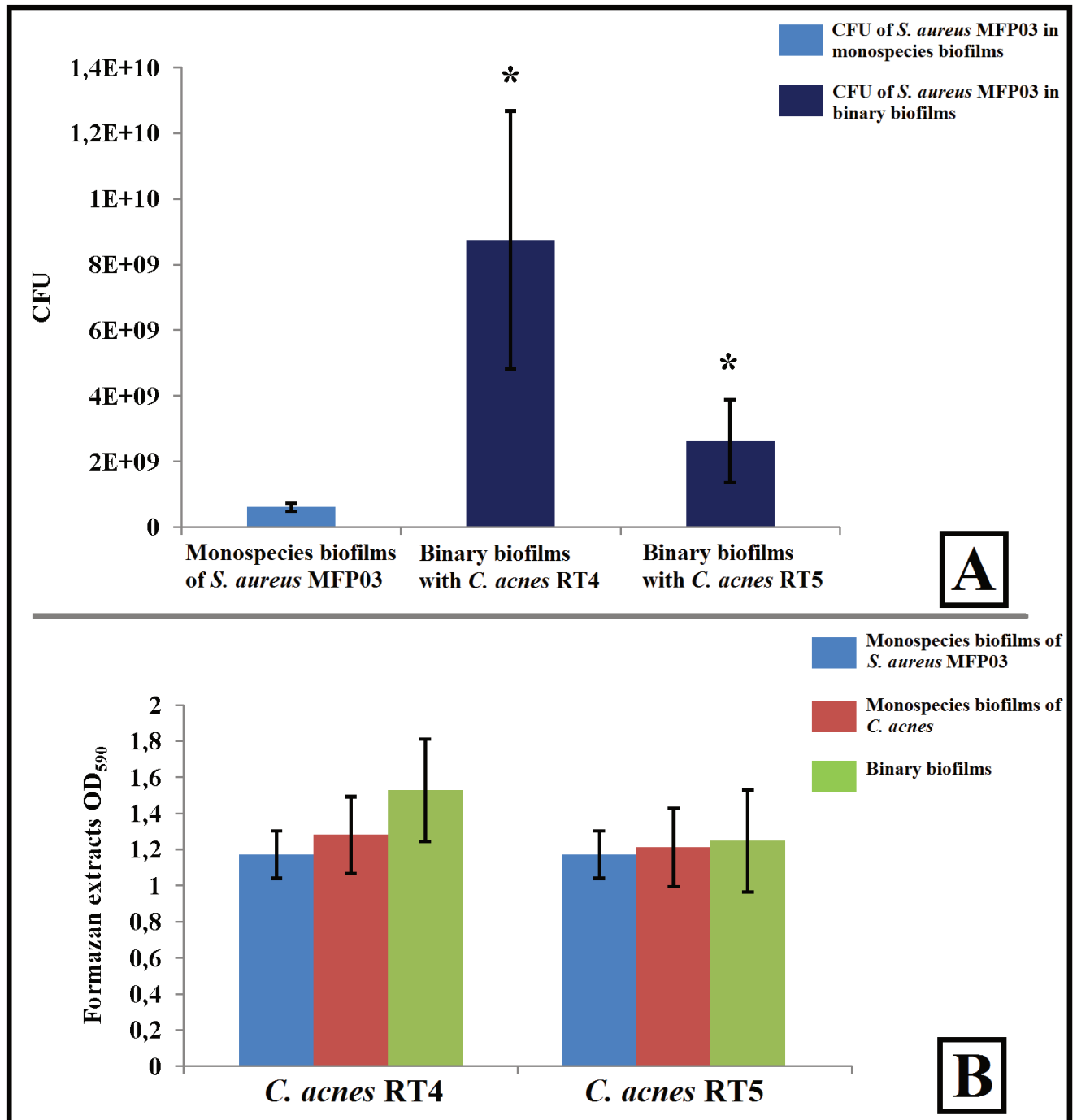


Figure 5.

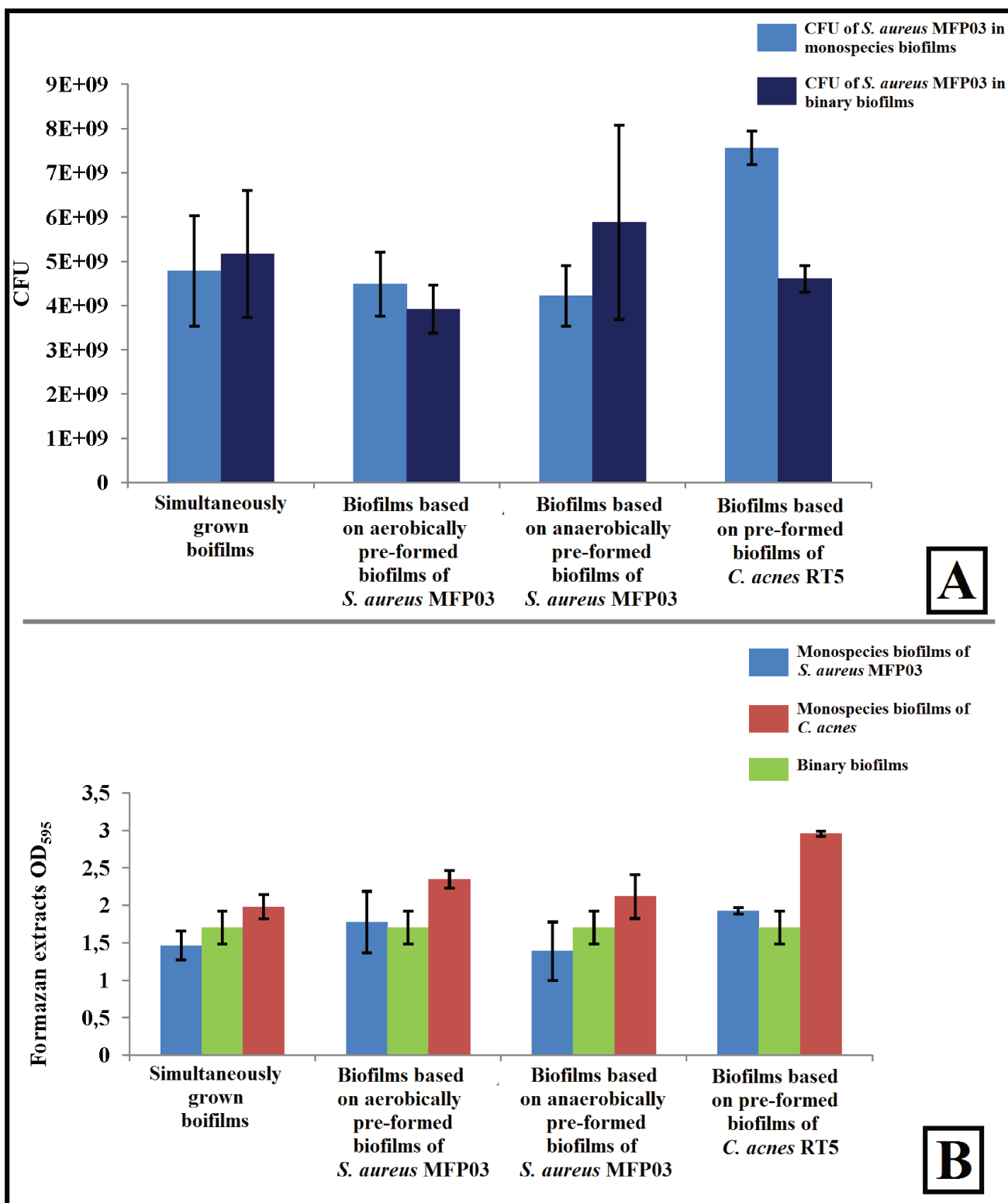


Figure 6.

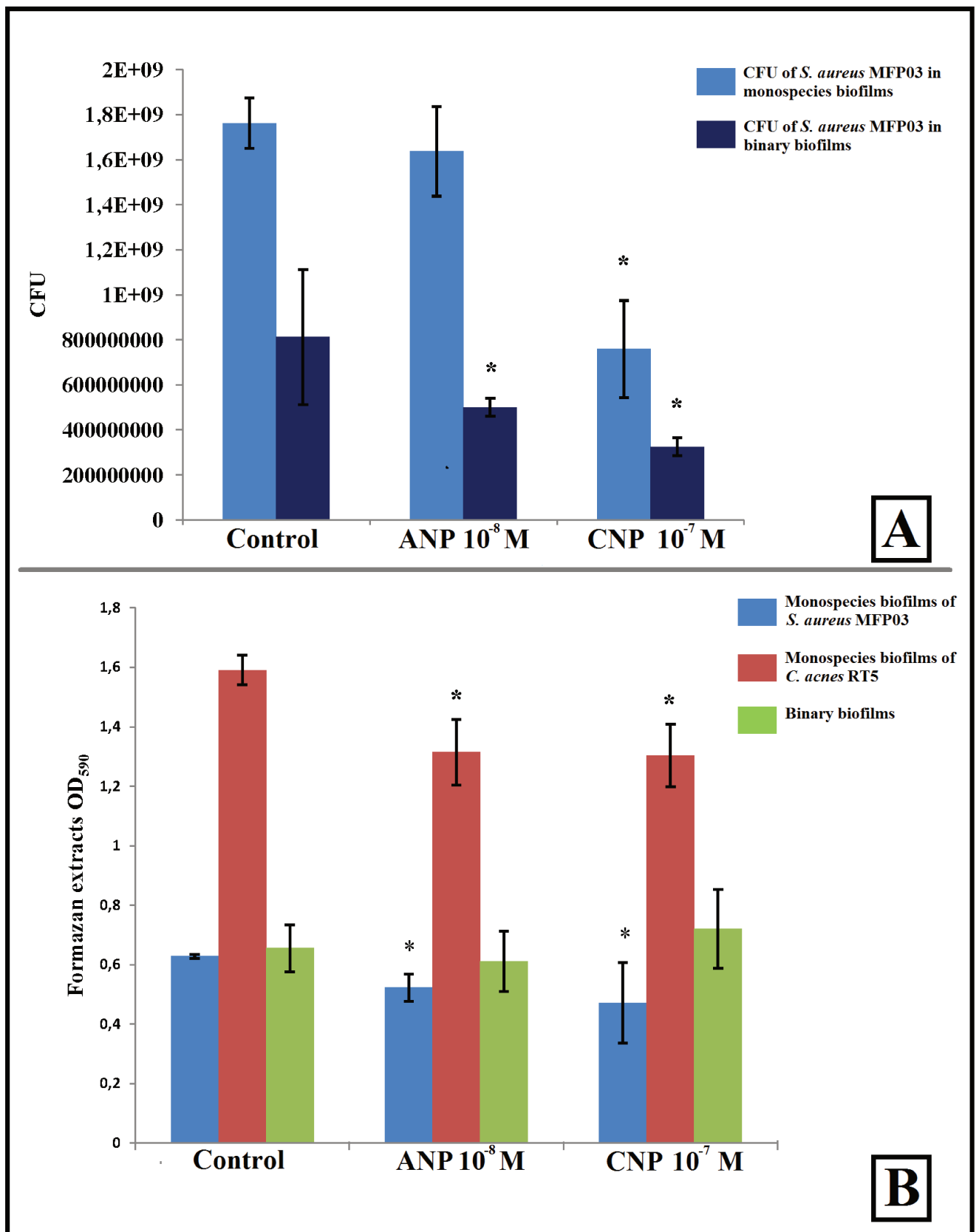


Figure 7.

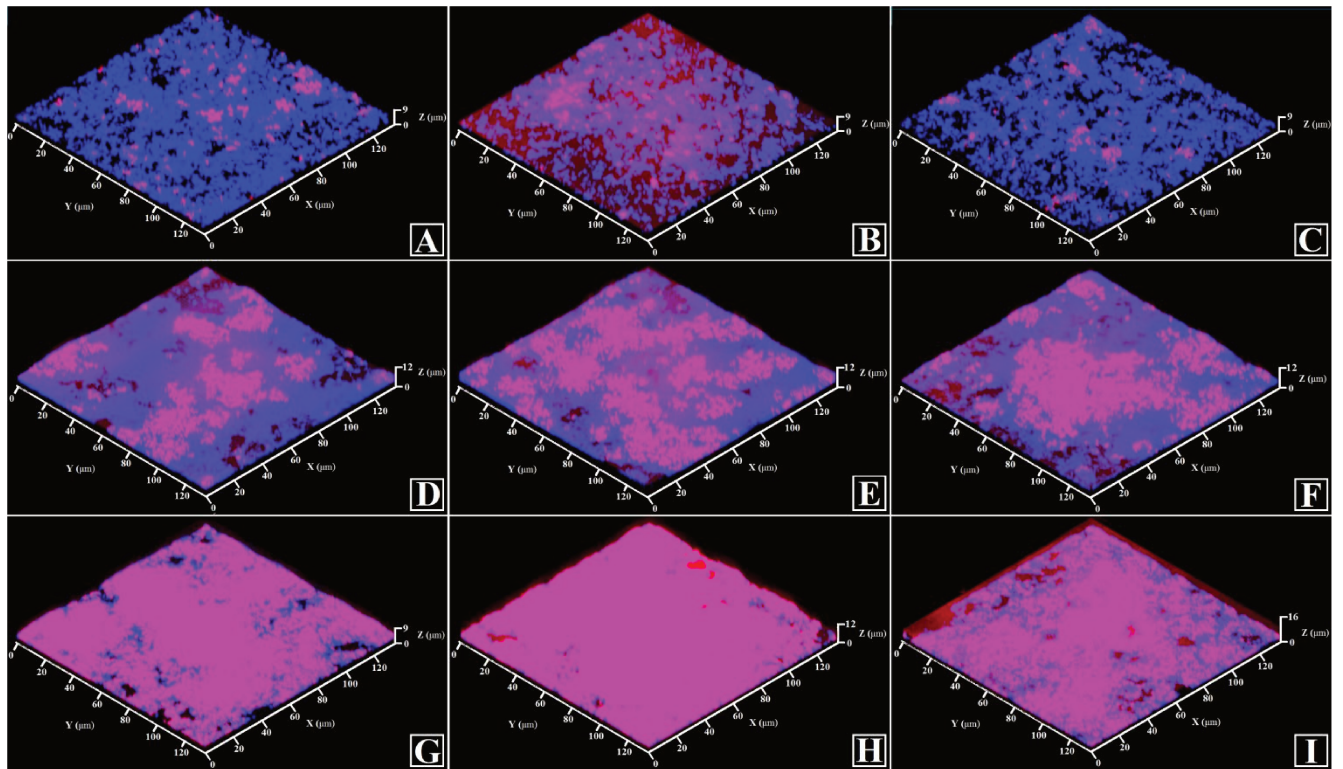


Figure 8.

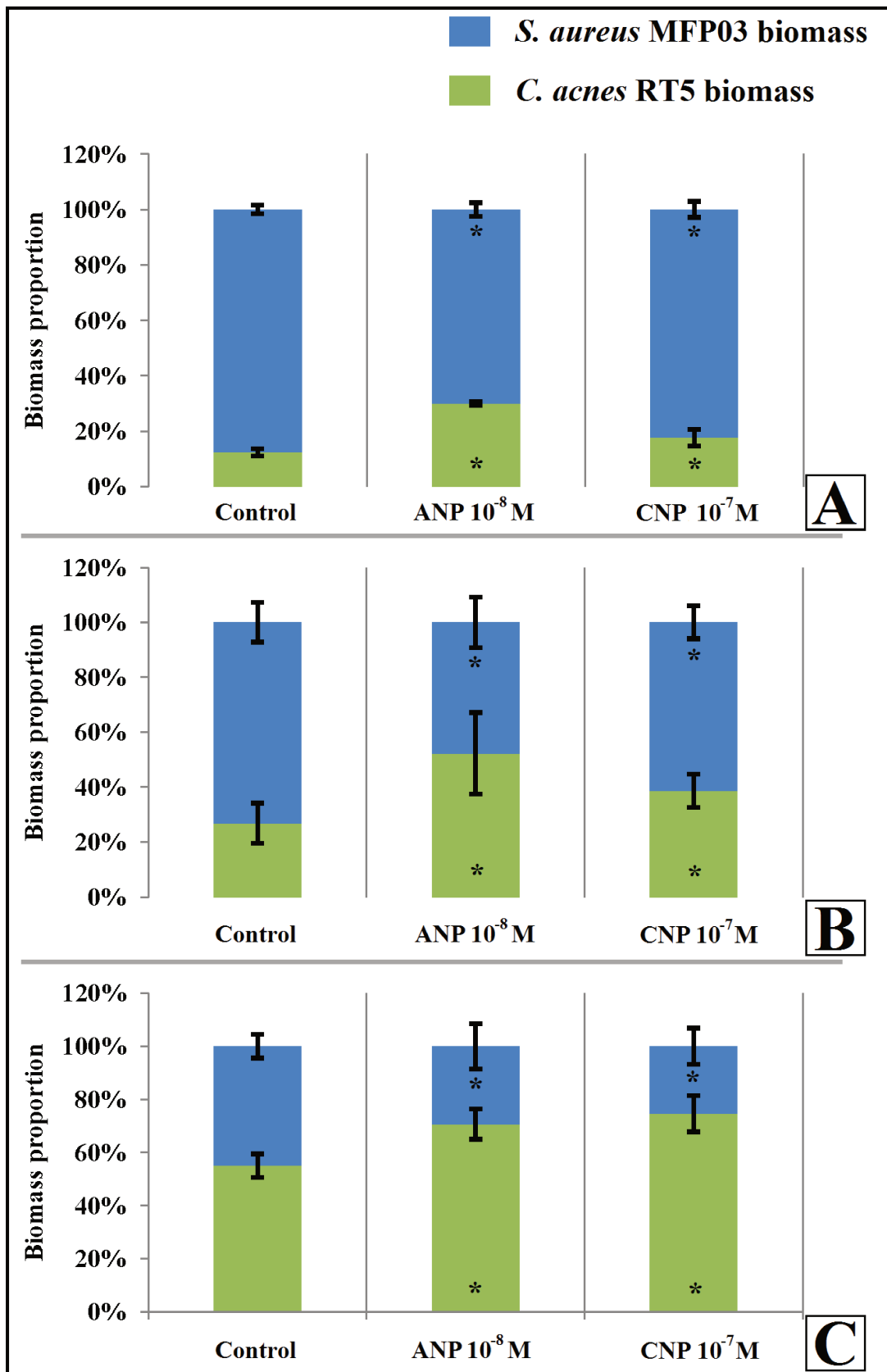


Figure 9.

### Composition of biofilm matrix of *Cutibacterium acnes* acneic strain RT5.

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### Abstract

*Cutibacterium acnes* is an abundant skin commensal microorganism considered to be an opportunistic pathogen and causative of acne vulgaris and other disorders. *C. acnes* forms biofilms inside skin glands hollows, that is a potential cause of inflammation and exacerbations of skin disorders. Extracellular matrix is an essential component of microbial biofilms which performs a number of functions i.e. it acts like a diffusion barrier for different antimicrobial compounds. Data of matrix composition are important to develop new antibacterial drugs with high antibiofilm efficiency, which can penetrate the biofilm and reach the cells. Here, we developed the new technique for Gram-positive bacteria biofilm matrix extraction without use of chemical digestion or enzymes. We found that acneic *C. acnes* RT5 strain biofilm matrix consists essentially in polysaccharides and proteins. The ratio of the main matrix components was determined. We detected 447 proteins in the matrix, hydrolases, chaperons, hemolysis factors and more than 40 proteins of unknown function. These data shows the complexity of matrix composition and make prospects for *C. acnes* biofilm further investigations and potential anti-biofilm drugs development.

**Keywords:** *Cutibacterium acnes*, biofilms, biofilm matrix, Orbitrap mass-spectrometry

### Introduction

Extracellular matrix is an integral feature of biofilms. It functions as a shield that protects bacteria from predators, participates in the formation of oxygen and signaling molecules gradients<sup>1</sup>. Also, the matrix protects against antibiotics and the immune system<sup>2</sup>. The extracellular matrix consists of polysaccharides, proteins and extracellular nucleic acids<sup>3</sup>. To date, the following functions are attributed to the biofilm extracellular matrix: structure, sorption, surface-activity, catalytic-activity, information, redox-activity and nutrition<sup>4</sup>. The structural role of the matrix is due to neutral polysaccharides and which form the carcass of the extracellular matrix. Such polysaccharides are synthesized by many bacteria, for example *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Enterobacter aerogenes*<sup>5,6</sup>. The sorption function of the matrix is carried by polymers whose task is the sorption of charged molecules like poly- $\gamma$ -glutamate (PGA<sup>7</sup>). The surface-activity of the matrix relies to the presence of biosurfactants. Biosurfactants are amphiphilic surfactant secondary metabolites: from low molecular weight glycolipids, sophorolipids, rhamnolipids, lipopeptides to high molecular weight proteins, lipopolysaccharides and lipoproteins<sup>8</sup>. The catalytic activity of the matrix is represented by enzymes that modify the matrix polysaccharides, matrix proteins and other components<sup>4</sup>. The redox-activity is due to electron-transport molecules like phenazines in *P. aeruginosa* which are able to act as antibiotics and virulence factors for eukaryotic cells<sup>9</sup>. Extracellular DNA performs informative, nutritional, structural, regulatory, protective and adhesive roles, therefore, taked part to the different functions of the matrix<sup>10</sup>.

The biofilm matrix acts as a strong barrier for different active compounds<sup>11</sup> and it is well demonstrated that bacteria in biofilms are particularly resistant to antibiotics and biocides. This is a major problem for eradication of pathogens and in this regard, determining the matrix composition is a key point to develop compounds with anti-biofilm activity. However, if the target is simple to define, the goal is difficult to achieve technically. Indeed, bacteria and matrix molecules are intimately associated with the growth medium and it is particularly delicate to extract matrix compounds without inducing a massive bacterial lysis or without artefact due to contamination by the growth medium. *Cutibacterium acnes*, former member of the genus *Propionibacterium*<sup>12</sup> is one of the most abundant microorganisms of the human skin. In some areas of the skin, it is practically the only microorganism isolated. *C. acnes* is closely related to humans, and its abundant presence on the skin may be a consequence of the positive effect of such coexistence<sup>13</sup>. *C. acnes* are gram-positive immobile, facultative anaerobic coryneomorphic rod-shaped bacteria<sup>14</sup>, preferring sebaceous and moist skin areas (forehead, neck, back<sup>14,15,16,17</sup>). Due to lipases, *C. acnes* can consume sebum and iscommonly found inside the hollows of the

sebaceous glands and hair follicles<sup>18</sup>. Currently *C. acnes* is considered as an opportunistic human pathogen associated with a variety of diseases. First of all, acne<sup>19</sup> of course, but also in association with a large variety of medical devices on which it can bind (shoulder implants<sup>19</sup>, cardiac implants<sup>20</sup>, contact lenses infections<sup>21</sup>). The frequency of shoulder implants due to *C. acnes* biofilms is particularly high<sup>19</sup>, presumably since *C. acnes* is common in those skin areas. The danger associated to *C. acnes* development on internal implants is due to the very long delay between the operation and the appearance of the clinical signs, up to several years<sup>19</sup>, because of the slow growth of this microorganism. *C. acnes* biofilms are also now considered as one of the reasons for the stability of *C. acnes* during acne antibiotic treatments<sup>23</sup>.

*C. acnes* biofilms are then of major interest but our knowledge of their matrix composition remains marginal. Previous studies have shown that the matrix of *C. acnes* biofilms contains DNA, proteins and polysaccharides<sup>20,22</sup>. However, these works raised a number of methodological concerns as, as previously mentioned, obtaining a clean biofilm matrix is a complex operation. There is also probably a certain strain related variability, and with the appearance of ribotyped *C. acnes* strains as references it is necessary to document this question..

### Materials and methods

***C. acnes* RT5 biofilm growth and preparation of biofilm biomass for matrix extraction.** 3-days cultures of *C. acnes* RT5 were washed twice with sterile physiological saline (pH 7.0) and the OD<sub>580</sub> was adjusted to 0.5. RCM-agar (25 mL) was distributed in 90 mm Petri dishes (PD). Two sterile hydrophilic 25 mm cellulose acetate filters (Sartorius) were placed in each PD. These filters were used as carriers to allow biofilm formation and biomass production in the absence of contamination by the medium. Then, 200 µL of a washed cell suspension was placed on each filter and distributed over the entire filter surface with a pipette tip. A total of 14 PD with filters were incubated together in a GasPack® anaerobic chamber at 37°C for 7 days. After incubation, 1.5 ml of wet biomass was harvested into 50 ml Falcon conical centrifuge tube (Corning) and mixed with 1 ml of sterile milliQ (MQ) water and homogenized by vortexing. After vortexing, in a first step, the biomass was centrifuged for 5 min at 2000 rpm at room temperature (RT). *C. acnes* is very resistant to sonication and in order to separate the matrix from the bacterial cells the biomass was sonicated either in a Branson Digital Sonifier 250 (USA) with 3/16" exponential pikes for 15 min with 25% amplitude (120 µm) and frequency 20 kHz, or in a Sonyprep 150 Plus (Great Britain) with 3 min exponential pikes for 10 min at a frequency 23 kHz and amplitude 150 µm.

***C. acnes* RT5 matrix extraction.** The matrix was then extracted from the biomass by



ultracentrifugation on caesium chloride gradient. The gradient was prepared in 26.3 ml Beckmann Coulter 355618 PC ultracentrifuge tube () by addition of 3 mL of  $\omega$  46%, 28%, 14% and 7% CsCl solutions (**Table 1**). Then, the suspension of sonicated biomass was layered on the top the CsCl gradient and submitted to centrifugation for 3h at 17,0000xg using a Beckmann Coulter Optima X-100 ultracentrifuge and an XXXXX rotor. At the end, the supernatant containing the matrix was collected and distributed in Corning centrifuge tube and centrifugated for 20 min at 15000xg to remove any residue of cells which are in the liquid after supernatant smpling from tube. After OD<sub>540</sub> of matrix samples was measured, and samples stored at -20°C.

Table 1. CsCl solutions used on gradient column (in order of layering).

Nº of CsCl solution	$\omega$ (CsCl), %	$d_{20}^{20}$ , g/ml
1	46	1,5158
2	28	1,2644
3	14	1,1163
4	7	1,054

**Evaluation of bacterial lysis by lactate dehydrogenase activity measurement.** Lactate dehydrogenase (LDH) is an intracellular enzyme<sup>24,25</sup>, thus it can be employed as an indicator of cell lysis during biofilm matrix extraction. and *C. acnes* normally express LDH (Counotte et al., 1980; Koniarová, 1993; Brzuszkiewicz et al., 2011). LDH-activity was measured using a commercial kit (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega, USA). Briefly, the method is based on the colorimetric measurement of formazan (OD<sub>490</sub>) formed during the conversion of lactate into pyruvate under the effect of LDH. In this reaction NADH is the source of electrons used for the transformation of iodidee-tetrazolium violet into formazan. *C. acnes* RT5 from centrifugation pellets were used as positive controls. The cell suspension was adjusted to the level of the matrix suspension OD, and the LDH activity was measured in both compartments.

**Complementary biofilm extraction tests** In order to evaluate the stability of *C. acnes* RT5 during biofilm matrix extraction, the biomass containing cells and matrix was submitted to was more harsh conditions, *i.e.*, by complementary treatment with LDH-assay kit lysis buffer (Promega, USA), lysozyme (Sigma), from and their combinations associated to sonication

(Table 2). After centrifugation as described elsewhere, the LDH-activity was measured in the samples. In parallel, an aliquot of each samples was layered on a microscopic slide, fixed with XXXX and stained by crystal violet (CV, 0.1%). The presence of bacterial cell bodies or fragments was examined by microscopy using a Zeiss Axio Observer A1 microscope

Table 2. Complementary biofilm extraction conditions tested (by order of harshness increment).

Condition №	Description
1	Intact biomass without treatment (control)
2	Biomass was treated with 1x lysis buffer (Promega) for 40 min at RT
3	Biomass was treated with 0.01 mg/ml lysozyme in 1x lysis buffer (Promega) for 30 min at 37°C
4	Cells were treated with 1 mg/ml lysozyme in buffer (0.1 M Tris-HCl + 0.05 M EDTA, pH 8.0) for 30 min at 37°C. After, biomass was sonicated in Branson Digital Sonifier 250 (USA) with 3/16" exponential horn for 15 min with 50% amplitude (229 µm) and frequency 20 kHz
5	Cells were treated with 2 mg/ml lysozyme in buffer (0.1 M Tris-HCl + 0.05 M EDTA, pH 8.0) for 30 min at 37°C. After biomass was precipitated, pellet was resuspended in 5x lysis buffer (Promega) and incubated for 40 min at RT. After, biomass was sonicated in Branson Digital Sonifier 250 (USA) with 3/16" exponential horn for 15 min with 50% amplitude (229 µm) and frequency 20 kHz in presence of glass beads

**Quantification of total organic carbon in *C. acnes* RT5 biofilm matrix.** The total organic carbon in the matrix was measured by use of a wet mineralization method. Organic matter was mineralized by a method adapted from Walkley, 1947<sup>26</sup> using a mix of concentrated sulfuric acid (SA, Sigma) and potassium dichromate (Sigma) . Briefly, 14.71 g of dichromate were dissolved in 150 mL of MQ-water. After that, 100 mL of 96% SA were added. Matrix samples were diluted 10 times and 1 mL of each diluted sample was mixed with 3 ml of reagent mix and incubated in a boiling water bath (100°C). In parallel, series of glucose solutions of different concentrations (10-2000 µg/mL in MQ-water) were prepared to make calibration curves. After boiling for 90 min, the samples were cooled, and the OD<sub>590</sub> was measured. Quantification of total organic carbon was made on the basis of a carbon mass proportion of 40%

in glucose (molecular mass of glucose is 180 g/mol, carbon part mass is 72 g/mol).

**Quantification of reducing sugars and proteins in the matrix.** The concentration of reducing sugars, as markers of total carbohydrates, was measured by the anthrone method (Dreywood, 1946). SA reacts with carbohydrates and resulting furfuroles condensate with anthrone with production of colored compounds. Briefly, 0.2 g of anthrone (Sigma) were dissolved in 100 mL of 96% SA (Sigma) to make the anthrone reagent. For calibration curves, standard glucose solutions were prepared. For that, 1 g of glucose (Sigma) was dissolved in 100 mL of benzoic acid solution made by dissolving 2.5 g benzoic acid in 1 L of MQ-water. Standard glucose solutions were maintained at 4°C in darkness. Before analysis, a partial hydrolysis of polysaccharides was performed to increase the concentration of reducing sugar groups. Matrix samples were diluted 50 to 80 in MQ-water. In glass tubes, 2 ml of each diluted sample were mixed with 200 µl of 96% SA and then heated for 4 h in a boiling water bath. After hydrolysis was over, samples were cooled to RT and a double volume of anthrone reagent was added to each sample. Samples were mixed and heated again at 100°C for 16 min. In parallel the same reaction as performed with series of glucose solutions (made from standard solution) with concentration ranging from 5 to 50 µg/mL. MQ-water was used as a negative control. After incubation the tubes were cooled to RT and OD<sub>625</sub> was estimated. Finally, the concentration of reducing sugars was calculated by use of the calibration curve.

Proteins were measured using the Bradford method as described in Bradford, 1976<sup>27</sup>. Standard Bradford reagent (Sigma) and bovine serum albumin (BSA, Sigma) were used in these experiments.

**Detection of DNA concentration in *C. acnes* RT5 matrix.** DNA concentration in matrix was estimated by the Dische method<sup>28</sup>. This method is based on the reaction of desoxyribose with diphenylamine in acetic acid. When heated levulinic aldehyde is produced and condensate with diphenylamine to produce a colored compound. Briefly, to prepare diphenylamine solution 1 g of diphenylamine (Sigma) was dissolved in 100 mL of pure acetic acid (Emsure, Germany). Thereafter, 2.75 ml of 96% SA was added. After calibration series of standard DNA solutions 50-500 µg/mL were prepared using herring sperm DNA (Sigma), samples of matrix were prepared for analysis. All samples (2 mL) were distributed in glass tubes. Then, a double amount of Dische reagent (4 mL) was added and the samples were heated at 100°C in a water bath. OD<sub>595</sub> was measured after cooling. MQ-water was used as a control.

**Orbitrap mass-spectrometry investigation of *C. acnes* RT5 matrix proteome.** For orbitrap mass-spectrometry, protein samples from the matrix were prepared in a special way. First, matrix samples were submitted to dialysis in 0.1-0.5 kDa pore size bags (Spectrum

Repligen, USA) to remove CsCl. After, samples were mixed with 4x volume of acetone and incubated for 10 min at RT to precipitate proteins. After precipitation, samples were centrifuged at RT 15,000xg for 30 min to collect proteins. At all steps (before dialysis, after dialysis, liquid supernatants after proteins removal) a Bradford assay was realized to control protein content and evaluate the potential protein loss. Remaining liquid supernatants were transferred into 140 mm Petri dishes and acetone was evaporated at RT for 30 min. This remaining liquid without acetone was also tested for protein content by Bradford assay.

Direct preparation of the samples for analysis consisted in carrying out the following manipulations. Proteins were resuspended in 1 mL of acetone, transferred in Eppendorf tubes and precipitated by centrifugation at 13,000xg for 10 min at room temperature. The supernatant was then removed, the samples were dried in a SpeedVac evaporator for 15 min. If necessary, samples were stored at -20° C. Before analysis, the proteins were dissolved in 25 µl of R2D2 buffer consisting in: urea 7 M, thio-urea 2 M, tri-n-butylphosphine 5 mM, dithiothreitol 20 mM, 3-(4-heptyl)phenyl-3-hydroxypropyl dimethylammonium propyl sulfonate (C<sub>7</sub>BzO) 0.5 %, 3-[(3-cholamidopropyl) dimethylammonium]-1-propylsulfate hydrate (CHAPS) 2%. The proteins were then separated, digested with trypsin and sent for mass spectrometry.

Protein samples were mixed with SDS loading buffer (63 mM Tris-HCl, pH 6.8, 10 mM DTT, 2% SDS, 0.02% bromophenol blue, 10% glycerol), and loaded onto a SDS-PAGE stacking gel (7%). A short electrophoresis was performed (10 mA, 15 min). After migration, the gels were stained with Coomassie blue and de-stained with a solution containing 50% ethanol, 10% acetic acid and 40% deionized water. The revealed protein bands were excised, washed with water, and submitted to protein digestion with trypsin (2 µg per band). The digestion was achieved overnight at 37°C. Several steps of peptide extraction were performed in H<sub>2</sub>O/acetonitrile/trifluoroacetic acid (49.5/49.5/1). The peptides were then dried and stored at -20°C. For tandem mass spectrometry, all experiments were performed on a LTQ-Orbitrap Elite (Thermo Scientific) coupled to an Easy nLC II system (Thermo Scientific). One microliter of sample was injected onto an enrichment column (C18 PepMap100, Thermo Scientific). The separation was performed with an analytical column needle (NTCC-360/100-5-153, Nikkyo Technos, Japan). The mobile phase consisted in H<sub>2</sub>O/0.1% formic acid (FA) (buffer A) and CH<sub>3</sub>CN/FA 0.1% (buffer B). Tryptic peptides were eluted at a flow rate of 300 nL/min using a three-step linear gradient: from 2 to 40% B over 75 min, from 40 to 80% B in 4 min and 11 min at 80% B. The mass spectrometer was operated in positive ionization mode with capillary voltage and source temperature set at 1.5 kV and 275 °C, respectively. The samples were analyzed using the CID (collision induced dissociation) method. The first scan (MS spectra) was

recorded in the Orbitrap analyzer ( $R = 60,000$ ) with the mass range  $m/z$  400–1800. Then, the 20 most intense ions were selected for  $MS^2$  experiments. Singly charged species were excluded for  $MS^2$  experiments. Dynamic exclusion of already fragmented precursor ions was applied for 30 s, with a repeat count of 1, a repeat duration of 30 s and an exclusion mass width of  $\pm 10$  ppm. Fragmentation occurred in the linear ion trap analyzer with collision energy of 35 eV. All measurements in the Orbitrap analyzer were performed with on-the-fly internal recalibration (lock mass) at  $m/z$  445.12002 (polydimethylcyclsiloxane). Raw data were then imported in the Progenesis LC-MS software (Nonlinear Dynamics). For comparison, one sample was set as a reference and the retention times of all other samples within the experiment were aligned. After alignment and normalization, statistical analysis was performed for one-way analysis of variance (ANOVA) calculations. For quantitation, only peptide features presenting  $p$ -value  $< 0.05$ ,  $q$ -value  $< 0.05$  and power  $> 0.8$  were retained. MS/MS spectra from selected peptides were exported for peptide identification with Mascot (Matrix Science) used against the database restricted to *C. acnes*. Database searches were performed with the following parameters: 1 missed trypsin cleavage sites allowed; variable modifications: carbamido-methylation of cysteine and oxidation of methionine. For each growth condition, the total cumulative abundance of the protein was calculated by summing the abundances of peptides. Proteins presenting  $p$ -value  $< 0.05$ ,  $q$ -value  $< 0.05$  and power  $> 0.8$  were kept. Moreover, only proteins with a variation above 2-fold in their average normalized abundances between growth conditions were retained.

#### **Surface-enhanced Raman spectroscopy of *C. acnes* RT5 matrix**

Raman spectroscopy is a method for intact samples analysis which is based on the phenomenon of inelastic scattering of monochromatic light (laser) during interaction with molecules<sup>29</sup>. This allows us find out changes in whole biochemical composition of cell or biofilm matrix during different processes<sup>29,30</sup> – cultivation or acting of biologically active compounds. Also, it allows us indicate microorganisms in complex matrices like blood, sputum, different foods etc<sup>31</sup>. Raman scattering itself is weak for detecting. Thus, to make spectra obtaining easier, additional substrates which increase intensity of Raman spectra are used: metal coverage of glass, metal (Ag, Au) nanoparticles, tags, specially covered surface (positive-negative ion interaction, selective molecules – antibodies etc). This technique is called surface-enhanced Raman spectroscopy (SERS)<sup>31</sup>. We used golden nanoparticles as enhancing substrate.

. For SERS analysis intact biomass of biofilms, biomass after matrix extraction and biofilm matrix of *C. acnes* RT5 were used. Biomass (intact and after matrix extraction) of *C. acnes* RT5 was washed thrice with acetone and dried at RT. Before SERS analysis, dry samples were washed twice with 0.9% NaCl solution: pellets were resuspended and centrifuged at 3000xg and

RT for 10 min.

For SERS gold nanoparticles were used. Nanoparticles were synthesized according to Bastus et al. with modifications<sup>32</sup>. After synthesis of golden seeds for nanoparticles, 40  $\mu\text{L}$  of 25 mM of  $\text{HauCl}_4$  and 40  $\mu\text{L}$  of 60 mM sodium citrate. Were mixed in tube cooled to 15°C. The tubes were closed and then incubated for 30 min at 90°C in a water bath. After cooling at to 15°C, 2 mL of suspension were removed from the tube, and 1.5 mL of deionized water were added. After the third generation of nanoparticles from basic precursors (tetrachloroaurate, sodium citrate), for the next generations during each step 60  $\mu\text{L}$  of 25 mM sodium hydroxide were added to the mix. Synthesis was conducted until obtaining 12-th generation nanoparticles.

Nanoparticles were characterized by transmission electron microscopy (TEM) and screening electron microscopy (SEM). For TEM, 5  $\mu\text{L}$  of nanoparticles suspension were placed on a TEM grid (Ted Pella, Redding, USA) and dried at RT. TEM images were obtained using a JEM-2100 microscope (Jeol, Japan) in acceleration voltage 200 kV. SEM images were obtained using a JIB-4501 microscope (Jeol, Japan).

SERS-spectra were obtained in using a BWS415 spectrophotometer (BWTEC, Germany). Samples were placed in XYZ-stage, laser position was controlled via an USB-microscope Mikmed-2000R (Micromed, Russia). To register SERS-signals different substrata based on golden nanomaterials were used.

SERS-spectra were processed using the GNU/Octave software. Briefly: spectra were imported from data files of the spectrophotometer, background fluorescence noise was eliminated by the polynomial fitting method. High-frequency noise were removed by use of developed filters. Peaks lists were exported in Excel software for visualization and interpretation. Filters were developed by use of the “signal” function of the GNU/Octave software with the following specifications: pass band 0.1 f, attenuation band 0.15 f, allowed oscillation less than 0.5 dB, filter attenuation 30 dB. A category II Chebyshev filter was used because of its lowest order, that is beneficial for calculation stability. After normalization, the spectrum of the nanoparticles was subtracted from the general spectra. Spectra were visualized in form of Excel graphs.

**Statistics.** Experiments were conducted at least in five independent repeats. Data were processed in Microsoft excel software. All numeric data were analyzed via Mann-Whitney nonparametric test. Numeric results are presented as average meanings with standard error of mean as error bars.

## Results



**Matrix extraction of *C. acnes* RT5.** After matrix extraction procedures and ultracentrifugation matrix was split into two phases (Fig. 1B): transparent upper phase of brownish shade (because of porphyrins) and turbid lower phase. OD<sub>540</sub> of upper and lower phases were in average 0.1 and 1.2 respectively.

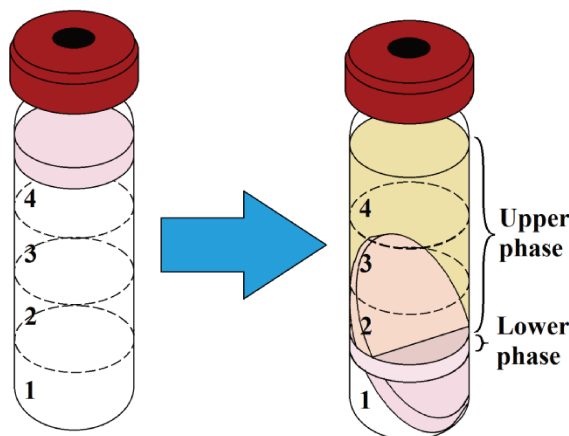


Figure 1. Ultracentrifugation of *C. acnes* RT5 prepared biofilm biomass and matrix extraction. Left : Gradient composing and inoculation of sonicated biomass. Right : Typical aspect of the two phases of the matrix after ultracentrifugation. N° 1,2,3,4 refer to the CsCl solutions as indicated in Table 1.

Measurement of the LDH-activity showed that both in the upper and lower phases the OD<sub>490</sub> of formazan was about 0.05. In samples of control intact bacteria, the OD<sub>490</sub> was 0, whereas in bacterial suspensions treated with lysis buffer it reached to 0.1. The higher LDH activity was measured only in samples of intact biomass (after ultracentrifugation), where the OD<sub>540</sub> of suspension was 1.0. In such samples OD<sub>490</sub> of formazan was about 1.5). This is showing that the LDH activity of *C. acnes* RT5 is low. While biofilm grows, a small part of cells is normally lysed and their contents diffuses into the matrix. That should explain the marginal amount of LDH detected in matrix samples.

In order to control that bacteria were not critically damaged during matrix preparation, *C. acnes* RT5 cells were treated with different lytic agents as indicated in Table 2.. In all the cases, the OD<sub>490</sub> measured after LDH assay was about 0.1 indicating that the membrane of *C. acnes* was not altered, even after exposition to lysozyme and ultrasounds. Moreover, cell aggregates were not dispersed (Fig. 2A-C), except after exposure to the hardest conditions (lysozyme+ultrasounds+glass beads) (Fig. 2E). Cell aggregates were also partly disorganized when the bacteria were exposed to a combination of lysozyme 1 mg/mL and ultrasounds but without glass beads (Fig. 2D).

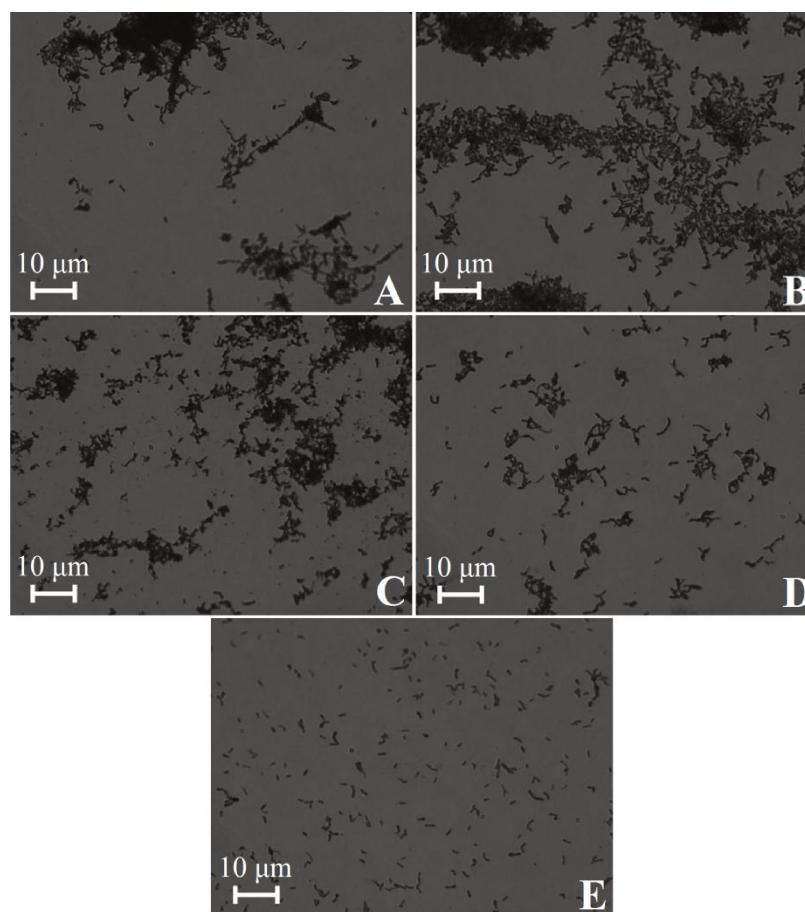


Figure 2. Light microscopy of *C. acnes* RT5 cell suspensions treated with different lytic protocols. A – Intact cells (control). B - Lysis buffer at RT. C - Lysozyme (0.01 mg/mL) + lysis buffer at 37°C. D - Lysozyme (1 mg/mL) + Lysis in buffer + ultrasounds. E - Lysozyme (2mg/mL) + Lysis buffer + ultrasounds in the presence of glass beads. (See Table 2 for further details)

**Quantitative biochemical analysis of basic matrix components of *C. acnes* RT5 biofilms.** By use of biochemical methods the proportion of basic *C. acnes* RT5 matrix components extracted by our method was quantified. Calculations were performed on the basis of total organic carbon amounts. Results are depicted on Figs. 3 and 4. The concentration of total organic carbon in the matrix was estimated to  $2.2 \pm 0.4$  mg/mL in the upper phase matrix and  $1.5 \pm 0.36$  mg/mL in the lower phase. According to the average volume of each phase (upper phase = 12 ml, lower phase = 3 ml), the mean concentration of organic carbon in the matrix appeared to account for  $2.1 \pm 0.4$  mg/mL. Herewith, the total mass of biofilm matrix extracted organic carbon was about 31.5 mg per 1,5 cm<sup>3</sup> of wet biofilm biomass. For each matrix component, the average carbon proportion in molecular mass was calculated. For carbohydrates the proportion of carbon in glucose is corresponding to 40% of the molecular mass. For proteins – on the basis of



the carbon proportion for a molecule of BSA it is of 45% of the molecular mass. For DNA we used an average proportion of carbon in 100 bp of *C. acnes* DNA taking into account its G-C pairs ratio, which is in about 60%<sup>33</sup>. This was leading to a value of 32%. The most abundant component in the matrix was the carbohydrate part. In the upper phase extract the amount of reducing sugars was  $3.7 \pm 0.76$  mg/mL (Fig. 3) or 64.3% of the total organic matter of the upper phase (Fig. 4A). In the lower phase the concentration of reducing sugars was  $1.44 \pm 0.46$  mg/mL (39.9% of the total organics matter of the lower phase, Fig. 4B). In total, in the matrix were extracted  $3.2 \pm 0.69$  mg/mL of reducing sugars corresponding to 54.2% of the total organics matter, Fig. 3, 4C), that is leading to a mean of 48 mg per 1,5 cm<sup>3</sup> of wet biofilm biomass.

The second part of the *C. acnes* RT5 matrix is the protein component. Bradford test showed a concentration of proteins in the upper phase of the matrix of  $307.6 \pm 61.2$  µg/mL (5.8% of the total organic matter, Figs. 3 and 4A), and  $531.8 \pm 248.7$  µg/mL (20.3% of the total organic matter, Figs. 3 and 4B) in the lower phase. Despite the fact that in the lower phase of the matrix heavy proteins are probably more abundant than in the higher part, their total amount accounts for less than the amount of light proteins if we take into consideration the volume of the two phases (35% versus 65%, respectively). From these assays and calculations, it appears that the general concentration of proteins in the matrix was about  $401.4 \pm 96.5$  µg/mL (11.9 % of the total organic matter of the matrix, Figs. 3 and 4C). Thus, the total mass of proteins in the matrix was about 6 mg per 1,5 cm<sup>3</sup> of wet biofilm biomass.

The concentration of DNA in the upper phase matrix extract reached  $139.8 \pm 21.4$  µg/mL (1.8% of all organics matter in the upper phase, Figs. 3 and 4A). In the lower phase it was  $274.9 \pm 76$  µg/mL (4.15% of total organic matter of the lower phase, Figs. 3 and 4B). The general concentration of DNA in the matrix was then about  $166.8 \pm 28.3$  µg/mL (2.79% of the total organic matter of the matrix, Figs. 3 and 4C), and the total mass of DNA was about 2.5 mg per 1-2 cm<sup>3</sup> of wet biomass. Then DNA appears as the polymeric component of matrix present in the lower concentration. On the other hand, this is showing that our method of matrix extraction was not damaging or destroying cells. As a complementary proof, we can mention that in experiments realized using a transformed strain of *Escherichia coli* ET12567 the mass of DNA obtained by the same technique from a biofilm of 0.1 cm<sup>3</sup> only revealed the presence of the plasmid pTetONCFPOpt<sup>34</sup> at a concentration of 100 µg/mL of the extract (*data not shown*).

By the method of wet combustion and calculation of proportions of all the major polymers in *C. acnes* RT5 matrix we observed that in the matrix there was generally 23.73% of organic carbon corresponding by difference to compounds which are not peptides, sugars, or DNA (Fig. 4C). In the upper and lower phases there molecules account for 23.26% and 26.53% of the total

organic carbon, respectively (Fig. 4A, 4B). Considering the metabolism of *C. acnes* we can hypothesize that these compounds should be essentially porphyrins and synthesis intermediates,. It should be noted that a qualitative reaction also revealed a big amount of  $\alpha$ -aminolevulinic acid (data not shown).

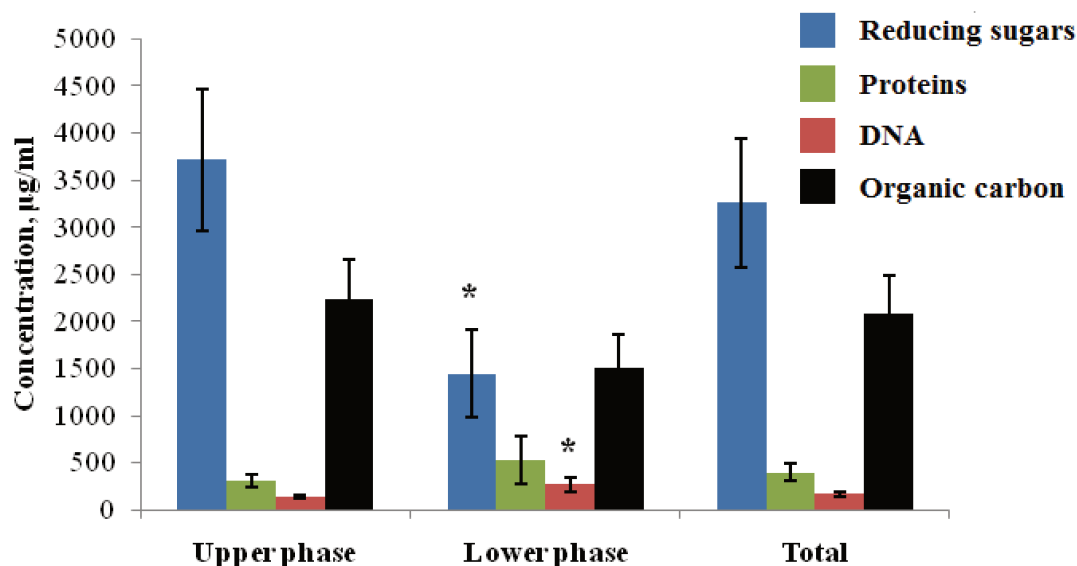


Figure 3. Concentration of basic matrix components of *C. acnes* RT5 biofilms, extracted by the present method.

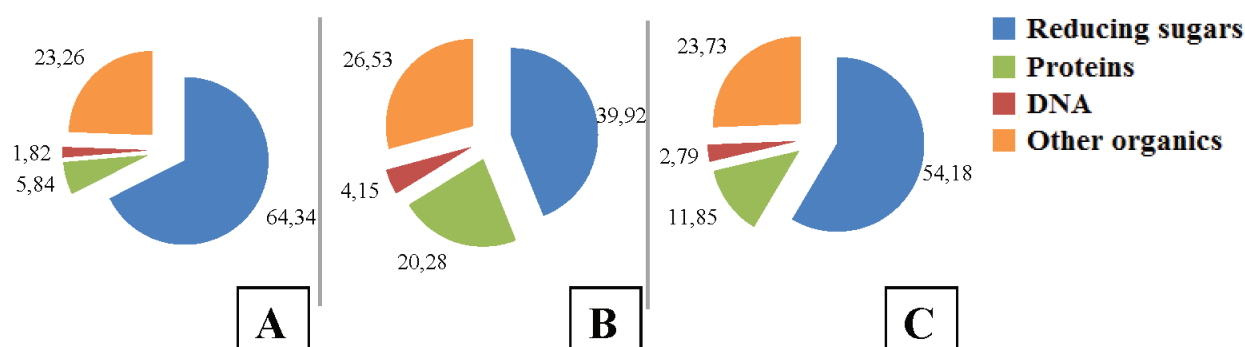


Figure 4. Proportion of the major components of the *C. acnes* RT5 biofilm matrix.

**Proteomics analysis of *C. acnes* RT5 matrix.** When proteins were prepared form Orbitrap analysis, Bradford tests were performed at each step to control the proteins loss. Before dialysis, the amount of proteins detected in the upper and lower phases was 19.5 µg/mL and 116.45 µg/mL, respectively. After dialysis, in the upper phase and in the lower phase there were 14.3 and 61.0 µg/mL of proteins corresponding to a protein mass of 28.57 and 305.19 µg, respectively. An important loss of proteins was then observed in the lower phase (-47.6%). Considering the pore diameter of the dialysis membrane that loss was probably affecting very short chain peptides.

Orbitrap proteomics analysis of *C. acnes* RT5 biofilm matrix revealed a total of 447

different proteins. 40 of these proteins were only present in the upper phase of the matrix extract, and 107 were only found in the lower phase (see Supplementary data 1, table 1), but this difference should be of limited significance, because most of the proteins including heavy enzymes were detected in both phases. A lot of these proteins were catalytic enzymes involved in the metabolism of sugars, proteins, and nucleotides. Many complex proteins were found in the matrix extract as different sub-parts (domains or monomers) of more complex molecular structures such as ribosomes or cytochromes. The presence of many intracellular enzymes can be explained by normal cell autolysis occurring in the biofilm during its formation and maturing. The same was for other non-enzymatic proteins. In matrix we found proteases, nucleases, enzymes of carbohydrates processing and 48 hypothetical proteins with unknown function. They should have different roles in matrix establishment or pathogenicity, but it is requiring deeper investigations. Also we found 43 ribosomal proteins in biofilm matrix (1 is putative).

Both phases contained the chaperonin GroL, a protein involved in stress response proteins folding. In *Escherichia coli* this protein can be localized into cytoplasm, but it can be found also in the membranes<sup>35</sup>. Next, we found three proteins of the DoxX family – 270, 210 and 133 amino acids in length. DoxX-family proteins include transmembrane proteins of unknown function, probably involved in sodium balance regulation in axonal membranes in eukaryotes<sup>36</sup>. It is not clear what are the functions of DoxX proteins in *C. acnes* RT5 biofilms too. In the matrix of *C. acnes* RT5 several established and putative hydrolases for different substrates were found, in particular two cellulases, another glycosyl hydrolase, hydrolases of nucleotides and an amidohydrolase, which all can play a role in biofilm formation and substrates consuming while biofilms are formed in skin. Especially glycerophosphodiester phosphodiesterase should be mentioned as an enzyme of lipids metabolism, that could be an important factor of *C. acnes* RT5 establishment and pathogenicity in skin. Putative HAD hydrolase, which was found in *C. acnes* RT5 matrix can be also involved in lipids cleavage<sup>37</sup>, as well as another putative hydrolase. Also, other possible hydrolases found in the matrix may be involved in the lipid metabolism. In addition to hydrolases, a number of enzymes of various intracellular processes have been discovered in the matrix of *C. acnes* RT5: catabolism of sugars, transcription and translation, cell cycle, synthesis of amino acids. Initiation, elongation and termination transcription factors have been also identified (see Supplementary data 1). It is worth also mentioning catalase and superoxide dismutase in the matrix that should participate in the protection to oxidative stress. Such molecules are essential for an anaerobic bacterium such as *C. acnes*, and theoretically could increase the resistance of *C. acnes* biofilms to unfavorable environmental factors.

It is interesting that in matrix of *C. acnes* RT5 the putative streptolysin associated protein

SagB was found. SagB is 343 amino acids protein (in comparison, *Streptococcus pyogenes* SagB contains 316 amino acids, AF067649<sup>38</sup>). In the *Streptococcus* genus SagB is a cytoplasmic cyclodehydrogenase that is involved in streptolysin SagA processing with use of flavin mononucleotide as electron acceptor<sup>39</sup>. Probably, in *C. acnes* RT5 this putative protein can be involved in similar process of other probable hemolysin processing that is important for pathogenicity. Also, it is interesting that in the lower phase the putative chaperone FliS was found. This is a protein involved in flagella synthesis in different motile bacteria<sup>40</sup>. The role of a putative FliS in a non-motile bacterium such as *C. acnes* is not obvious, but it should have other functions, maybe directly connected with the composition of the protein part of the biofilm matrix, because of it requires extracellular proteins folding and post-translation modification. CAMP-factors also should be included in our list of revealed proteins: hypothetical protein HMPREF9571\_02536 has more than 99% of similarity with CAMP-1 factor of other strains of *C. acnes* (according to databases Uniprot<sup>38</sup>, and NCBI<sup>41</sup>). It consist of 285 amino acids molecule of 30.394 kDa molecular weight. Next, at once two hypothetical proteins HMPREF9571\_00078 and HMPREF9571\_00834 are very similar to *C. acnes* CAMP-3 factor: they are 271 amino acids and have a mass of 29.1 and 29.25 kDa, respectively. A number of other hypothetical proteins also may be CAMP-factors.

### **Surface-enhanced Raman spectroscopy of the *C. acnes* RT5 biofilm matrix**

When analyzing the SERS spectra of the isolated matrix in comparison with the biomass before and after matrix isolation, more than 40 major peaks were revealed (see Fig. 5). Differences in peaks and their intensities serve as another proof that the matrix of the biofilm has been effectively isolated. Differences were established by visual determination and comparison of the height of peaks in the graph, peaks differed in height two or more times were considered different. Graphically, the peaks are shown in Fig. 5.

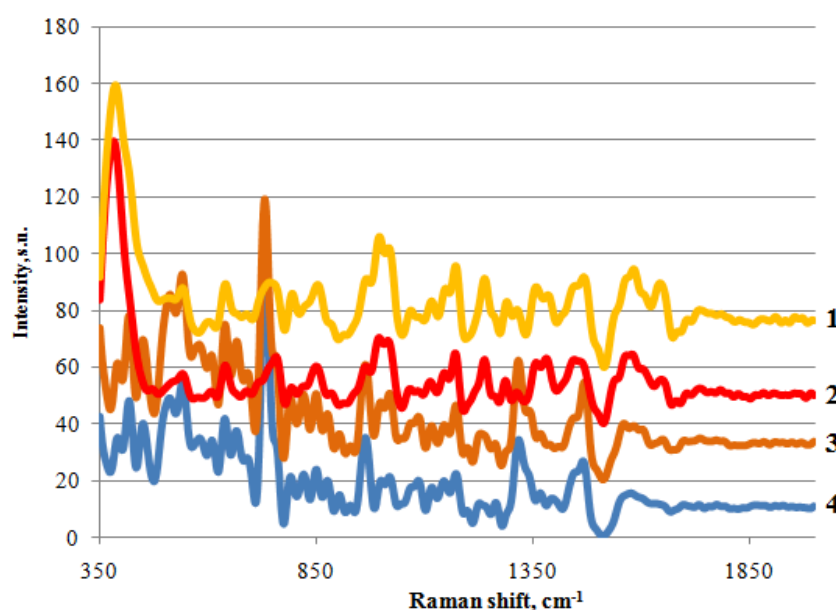


Figure 5. SERS spectra of biofilm biomass and biofilm matrix of *C. acnes* RT5. 1 – upper phase of matrix; 2 – lower phase of matrix; 3 – biomass after matrix removal; 4 – biomass of untreated biofilms; 2 -

The spectrum of each sample was plotted on a graph with a displacement along the ordinate axis to avoid superposition of the spectra. Biofilm total biomass spectra and matrix spectra are obtained, which in the future will be added to the database of microorganism spectra and will be used to identify *C. acnes* in complex biological objects and samples with the help of SERS. SERS has the advantages of a very simple sample preparation and fast results obtaining, so it is a promising tool for bacterial detection. It can be also noted that SERS-spectroscopy by itself is a potential powerful tool for detecting compounds as generally normally present in bacterial biofilm matrix. However, a more complete picture, of course, needs to be built using a combination of SERS with other methods but, to date, the weak point of SERS is the lack of a large spectra database of organic polymers, which greatly complicates the analysis. Then the present data essentially worth as a contribution to the formation of this first SERS data base of bacterial spectra.

## Discussion

Extracellular matrix is an essential part of microbial biofilms which conducts a number of functions and plays a key role in biofilm establishment and organization. Biofilms are difficult to eradicate also because of presence of matrix as a diffusion barrier for exogenous chemicals and biocides<sup>42</sup>. Understanding of matrix composition can be a key for biofilm properties prediction. It is perspective both for fundamental science and applied areas (biotechnology, pharmacology

and medicine). For biotechnology the data about biofilm matrix composition is necessary for developing optimal biofilm carriers to obtain optimal biofilm growth. Matrix physico-chemical properties based on its biochemical composition can be used in developing of materials of biofilm carriers. For example it is perspective for wastewater treatment systems which use anammox process and require a lot of anammox consortia biofilm biomass for optimal processing<sup>43</sup>. In medicine understanding of matrix composition can be a key for targeting of biofilms of pathogens.

Concerning targeting of biofilm matrix of mature biofilms, we can mention two possible strategies of biofilm eradication. For both of those strategies the knowing of matrix composition is absolutely essential. The first can be based on direct matrix disintegration with chemicals or enzymes. It can be used in *in vitro* or in cases of biofilms in artificial systems, but it also can make a financial challenge. For *in vivo* application it can also be a challenge because of possible damaging of host tissues. The second strategy can be based on matrix penetration without its significant chemical modification or destruction, which can be more perspective in *in vivo* application. Thus, it is necessary to find or develop antibiofilm compounds which can penetrate the matrix and reach microbial cells in biofilms or which can support traditional drugs transport through the matrix (nanovesicles etc.). In case of acne vulgaris it can be possible to develop, for example, drugs of cutaneous external use which can penetrate matrix of *C. acnes* biofilms in inflamed comedones and eradicate *C. acnes*.

At the time of this article writing, two papers on the composition of the *C. acnes* matrix have been published. In the first of them<sup>22</sup>, the matrix was studied using specific fluorescent dyes for DNA, polysaccharides, and proteins. Using fluorescent microscopy, the authors determined the presence of carbohydrates, proteins and DNA in the matrix without further deepening into the biochemical structure of the compounds. Another work<sup>20</sup> was devoted, among other things, to the isolation of the matrix of a *C. acnes* strain and the analysis of its composition. The authors estimated the amount of DNA, proteins and poly-N-acetylglucosamine (surface adhesin). Their data are close to those obtained in the present work. The authors also characterized some matrix proteins, for example, hydrolases, enolase, lipoprotein RlpA and hemolysis factor CAMP.

In the present work, a deeper and more comprehensive study of the *C. acnes* RT5 matrix was carried out. Firstly, we produced fully mature 7-day biofilms<sup>44</sup> on the surface of filters on a solid medium, which eliminated the material of the nutrient medium and made it possible to isolate the real biofilm matrix. We did not work with liquid media as it was in previous technique<sup>44,45</sup>, that allowed us to get pure biofilm without any planktonic cells. Secondly, by difference with previous studies we were not obliged to treat the biofilm with dispersin B and

DNase, which automatically deprives of the possibility to detect a large subset of the matrix components. Third, the technique presently developed allow to avoid any chemical alteration of the biofilm matrix before analysis. Then, for the first time we had access to the relative quantitative ratio of the main components of *C. acnes* matrix.

In the matrix of *C. acnes* RT5, most of the organic carbon (54%) belongs to the carbohydrate component. The second component is peptides: 11.9%. DNA was about 2.8% of the total organic matter of the matrix. The remaining organic matter, presumably, contains metabolites and intermediates, presumably involved in porphyrins synthesis. Analysis of the proteomatic matrix of the biofilm showed that it contains more than 400 proteins. Enzymes are very diverse, which indicates the high catalytic potential of the *C. acnes* matrix. Many of the proteins are proteins of the cytosol and probably enter the matrix following autolysis of a part of the cells, as normally occurring during the growth of biofilms. However, there is a high probability that these intracellular proteins within the matrix have certain unknown functions that are different from those inside the cell and are not a "dead weight". For example, several dozen hydrolases, which are specific for different substrates, probably allow *C. acnes* biofilms to dissolve polymers of surrounding tissues of the host organism, in particular, human skin, which causes their pathogenicity and probably determines the role of *C. acnes* in the development of acne and other diseases. Chaperones and chaperonins can participate in the organization of the matrix carcass, together with ribosomal particles and whole ribosomes. We do not know in what form the ribosomes are represented in the matrix: in the form of whole particles, in the form of separate subunits, protein aggregates or proteins separately. Moreover, out of more than 50 ribosomal proteins we detected only 43. We can assume that the ribosomes are represented in the matrix in all states, however, their potential role and the role of their proteins in the matrix of biofilms is still unclear.

SERS method was proven as simple and robust approach for biofilm matrix detection and spectra obtaining. As it was noted before<sup>29,30,31</sup>, it requires minor sample preparation and fast data obtaining. We conducted the first SERS analysis of a *C. acnes* biofilms matrix. Analysis of the SERS peaks also confirms the isolation of the biofilm matrix, since more than 40 peaks in the matrix and biomass of the cells are distinguished. The difference in peaks is an additional evidence of matrix isolation. The most intense peaks of both phases of the matrix were found at 370-386 cm<sup>-1</sup>. However, the spectra of SERS alone do not carry information about the nature of the matrix substances, since there is currently no SERS database of organic compounds and in particular of organic polymers. Therefore, SERS is advantageously used in combination with other techniques, such as mass spectrometry and NMR. Identification of spectra peaks requires



spectra obtaining for each compound of matrix. Also, SERS is a rather weak tool for identification of molecules of low concentration. Nevertheless, we can state that we first made typing of biofilms and *C. acnes* matrix by the SERS method, it will help in the future, to determine the presence of *C. acnes* in complex biological samples by the comparatively fast and easy SERS method. Also, it will be used in microorganism SERS-spectra database construction. This database will allow to identify microbes fast and easy without implementation of molecular biology approaches like PCR or fluorescence *in situ* hybridization.

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Table 3. Proteins identified in the matrix of *C. acnes* RT5 biofilms. The proteins isolated only in the lower phase of the matrix are labelled red, and the proteins in the upper are in blue.

Accession	Description	MW [kDa]	Coverage	# AAs
327443705	bacterial NAD-glutamate dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	172,238	3,82	1569
327446964	DNA-directed RNA polymerase, beta' subunit	143,329	10,67	1293

	[ <i>Cutibacterium acnes</i> HL043PA2]			
327445677	oxoglutarate dehydrogenase (succinyl-transferring), E1 component [ <i>Cutibacterium acnes</i> HL043PA2]	136,695	3,24	1236
327450411	pyruvate synthase [ <i>Cutibacterium acnes</i> HL043PA2]	131,812	12,13	1204
327446963	DNA-directed RNA polymerase, beta subunit [ <i>Cutibacterium acnes</i> HL043PA2]	131,21	7,17	1185
327445932	ribonucleoside-diphosphate reductase, adenosylcobalamin-dependent [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	103,873	8,84	950
327443701	preprotein translocase, SecA subunit [ <i>Cutibacterium acnes</i> HL043PA2]	101,327	2,9	898
327445767	alanine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	95,7476	5,49	892
327445892	aconitate hydratase 1 [ <i>Cutibacterium acnes</i> HL043PA2]	96,3455	3,49	888
327447637	pyruvate, phosphate dikinase [ <i>Cutibacterium acnes</i> HL043PA2]	96,4148	25,93	883
327444294	membrane alanyl aminopeptidase [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	96,6945	4,25	870
327444263	Anticodon-binding domain protein [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	97,2608	1,84	870
327449698	ATPase family associated with various cellular activities (AAA) [ <i>Cutibacterium acnes</i> HL043PA2]	93,2526	2,73	844
327444364	phenylalanine--tRNA ligase, beta subunit [ <i>Cutibacterium acnes</i> HL043PA2]	89,1879	2,98	839
327446071	leucine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	92,3907	5,45	826
327442870	NADH dehydrogenase (quinone), G subunit [ <i>Cutibacterium acnes</i> HL043PA2]	86,5539	3,33	812
327446134	S1 RNA binding domain protein [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	88,6422	3,11	803
327444160	guanosine pentaphosphate synthetase	78,8514	13,64	733

	I/polyribonucleotide nucleotidyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]			
327445140	methylmalonyl-CoA mutase large subunit [ <i>Cutibacterium acnes</i> HL043PA2]	80,1163	22,22	729
327446014	succinate dehydrogenase or fumarate reductase, flavoprotein subunit [ <i>Cutibacterium acnes</i> HL043PA2]	78,0746	5,35	710
327444579	translation elongation factor G [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	76,5564	9,47	697
327445877	threonine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	77,2639	6,82	689
327444113	succinate dehydrogenase or fumarate reductase, flavoprotein subunit [ <i>Cutibacterium acnes</i> HL043PA2]	75,7144	12,59	675
327446943	2-oxoacid:acceptor oxidoreductase, alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	70,328	7,18	655
327449725	ferrochelatase [ <i>Cutibacterium acnes</i> HL043PA2]	70,614	11,54	650
327445141	methylmalonyl-CoA mutase, small subunit [ <i>Cutibacterium acnes</i> HL043PA2]	69,0733	32,7	636
327444300	AMP-binding enzyme [ <i>Cutibacterium acnes</i> HL043PA2]	69,5205	5,83	635
327448387	GTP-binding protein TypA [ <i>Cutibacterium acnes</i> HL043PA2]	68,4269	5,48	620
327447627	chaperone protein DnaK [ <i>Cutibacterium acnes</i> HL043PA2]	66,3225	41,49	617
327449464	glutamine-fructose-6-phosphate transaminase (isomerizing) [ <i>Cutibacterium acnes</i> HL043PA2]	66,994	8,13	615
327449171	AMP-binding enzyme [ <i>Cutibacterium acnes</i> HL043PA2]	66,2291	11,53	607
327445770	aspartate--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	66,4973	18,98	606
327450412	pyridine nucleotide-disulfide oxidoreductase [ <i>Cutibacterium acnes</i> HL043PA2]	66,4245	12,23	605

327444157	thiamine pyrophosphate enzyme, N-terminal TPP binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	64,089	13,9	597
327444184	translation initiation factor IF-2 [ <i>Cutibacterium acnes</i> HL043PA2]	62,9378	6	583
327447714	arginine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	63,9594	31,1	582
327444193	proline--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	63,6595	32,36	581
327446077	malic enzyme, NAD binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	61,7616	26,23	568
327446586	ABC transporter, ATP-binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	61,9042	5,49	565
327443867	CTP synthase [ <i>Cutibacterium acnes</i> HL043PA2]	60,6502	8,01	562
327444155	hypothetical protein HMPREF9571_02286 [ <i>Cutibacterium acnes</i> HL043PA2]	60,8078	3,95	557
327449770	phosphoenolpyruvate-protein phosphotransferase [ <i>Cutibacterium acnes</i> HL043PA2]	57,6929	6,46	557
327449865	nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	58,6391	5,58	556
327445697	ATP synthase F1, alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	58,872	24,22	545
327449885	chaperonin GroL [ <i>Cutibacterium acnes</i> HL043PA2]	56,8047	44,3	544
327445845	phosphoglucosyltransferase, alpha-D-glucose phosphate-specific [ <i>Cutibacterium acnes</i> HL043PA2]	58,451	7,55	543
327449490	chaperonin GroL [ <i>Cutibacterium acnes</i> HL043PA2]	56,3943	62,52	531
327444272	trigger factor [ <i>Cutibacterium acnes</i> HL043PA2]	57,6801	16,42	530
327444120	signal recognition particle protein [ <i>Cutibacterium acnes</i> HL043PA2]	57,0852	10,8	528
327448391	methylmalonyl-CoA carboxyltransferase 12S	56,4265	33,97	524

	subunit [ <i>Cutibacterium acnes</i> HL043PA2]			
327447533	GMP synthase (glutamine-hydrolyzing) domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	55,5965	10,64	517
327449145	phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase [ <i>Cutibacterium acnes</i> HL043PA2]	54,9575	12,98	516
327446782	glycerol kinase [ <i>Cutibacterium acnes</i> HL043PA2]	56,8473	13,2	515
327445108	NAD(P)(+) transhydrogenase (AB-specific), alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	54,0842	3,88	515
327450419	iron-sulfur cluster-binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	56,9632	5,68	511
327445925	RNA polymerase sigma factor RpoD [ <i>Cutibacterium acnes</i> HL043PA2]	55,6427	3,72	511
327445032	cytosol aminopeptidase family, catalytic domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	52,0522	25,98	508
327446016	glycine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	57,1606	11,66	506
327447643	succinate CoA transferase [ <i>Cutibacterium acnes</i> HL043PA2]	55,0555	15,28	504
327447528	inosine-5'-monophosphate dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	53,6595	24,01	504
327445824	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase, A subunit [ <i>Cutibacterium acnes</i> HL043PA2]	53,0335	8,38	501
327447686	cellulase (glycosyl hydrolase family 5) [ <i>Cutibacterium acnes</i> HL043PA2]	54,0388	5,6	500
327446207	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase [ <i>Cutibacterium acnes</i> HL043PA2]	52,2162	5,21	499
327445083	aminopeptidase P domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	55,9833	14,46	498
327446180	putative ribosomal protein S1 [ <i>Cutibacterium acnes</i> HL043PA2]	55,1189	25,1	498



327448392	conserved carboxylase domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	54,6555	28,37	497
327445823	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase, B subunit [ <i>Cutibacterium acnes</i> HL043PA2]	54,2511	9,46	497
327450434	lysine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	55,2002	4,66	494
327445089	cellulase (glycosyl hydrolase family 5) [ <i>Cutibacterium acnes</i> HL043PA2]	53,0902	5,07	493
327449790	glycine hydroxymethyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	52,9026	14,66	491
327445699	ATP synthase F1, beta subunit [ <i>Cutibacterium acnes</i> HL043PA2]	53,4429	40,49	489
327445715	putative UDP-N-acetylglucosamine 1- carboxyvinyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	53,7019	11,29	487
327446637	succinate-semialdehyde dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	51,8836	6,39	485
327446613	catalase [ <i>Cutibacterium acnes</i> HL043PA2]	54,3092	4,97	483
327444245	FeS assembly protein SufB [ <i>Cutibacterium acnes</i> HL043PA2]	53,4615	14,73	482
327448376	adenylosuccinate lyase [ <i>Cutibacterium acnes</i> HL043PA2]	52,1836	7,76	477
327445802	glycosyl hydrolase, family 1 [ <i>Cutibacterium acnes</i> HL043PA2]	55,121	3,57	476
327451109	argininosuccinate synthase [ <i>Cutibacterium acnes</i> HL043PA2]	51,8299	14,14	474
327445058	glutamine synthetase, type I [ <i>Cutibacterium acnes</i> HL043PA2]	53,2083	17,76	473
327449394	glutamate--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	53,6473	9,11	472
327446201	UDP-N-acetylmuramate--L-alanine ligase [ <i>Cutibacterium acnes</i> HL043PA2]	49,4491	15,71	471



327446797	fumarate hydratase, class II [ <i>Cutibacterium acnes</i> HL043PA2]	50,7034	12,18	468
327443916	argininosuccinate lyase [ <i>Cutibacterium acnes</i> HL043PA2]	50,4988	10,9	468
327446003	putative alpha,alpha-trehalose-phosphate synthase (UDP-forming) [ <i>Cutibacterium acnes</i> HL043PA2]	52,8332	11,78	467
327445713	dihydrolipoyl dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	49,6976	16,27	467
327449929	UTP--glucose-1-phosphate uridylyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	51,1487	13,55	465
327446545	pyridine nucleotide-disulfide oxidoreductase [ <i>Cutibacterium acnes</i> HL043PA2]	49,6044	7,39	460
327449174	pyridine nucleotide-disulfide oxidoreductase [ <i>Cutibacterium acnes</i> HL043PA2]	48,0198	4,58	459
327445033	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	47,5648	29,19	459
327445885	putative tryptophan 2,3-dioxygenase [ <i>Cutibacterium acnes</i> HL043PA2]	50,7493	3,71	458
327446190	pyruvate kinase [ <i>Cutibacterium acnes</i> HL043PA2]	49,7848	33,62	458
327445732	ribosome biogenesis GTPase Der [ <i>Cutibacterium acnes</i> HL043PA2]	49,7877	5,26	456
327445776	histidine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	50,2328	9,03	454
327443723	putative hydrolase [ <i>Cutibacterium acnes</i> HL043PA2]	49,9688	4,42	453
327448394	PAS domain S-box protein [ <i>Cutibacterium acnes</i> HL043PA2]	49,1598	14,16	452
327449180	TQXA domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	47,897	5,97	452
327446616	putative aspartate ammonia-lyase [ <i>Cutibacterium acnes</i> HL043PA2]	49,1785	21,51	451
327449458	phosphoglucosamine mutase [ <i>Cutibacterium acnes</i> HL043PA2]	47,5134	18,22	450

	HL043PA2]			
327450255	amine oxidase (flavin-containing) [ <i>Cutibacterium acnes</i> HL043PA2]	47,5902	8,02	449
327443351	peptidase dimerization domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	47,1711	14,32	447
327449220	nicotinate phosphoribosyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	47,3962	13,9	446
327449724	glutamyl-tRNA <sup>Glu</sup> reductase domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	47,105	17,04	446
327445849	hypothetical protein HMPREF9571_01585 [ <i>Cutibacterium acnes</i> HL043PA2]	48,4552	29,66	445
327444207	Glu/Leu/Phe/Val dehydrogenase, dimerization domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	48,1119	18,65	445
327445963	amidohydrolase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	47,6689	6,29	445
327442869	NADH oxidoreductase (quinone), F subunit [ <i>Cutibacterium acnes</i> HL043PA2]	48,5162	11,71	444
327446834	putative YcaO-like family [ <i>Cutibacterium acnes</i> HL043PA2]	48,3883	12,44	442
327445680	homoserine dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	46,6288	14,29	441
327444166	glycine betaine/L-proline transport ATP binding subunit [ <i>Cutibacterium acnes</i> HL043PA2]	48,225	13,21	439
327449142	3,4-dihydroxy-2-butanone-4-phosphate synthase [ <i>Cutibacterium acnes</i> HL043PA2]	46,6248	16,67	438
327449718	glutamate-1-semialdehyde-2,1-aminomutase [ <i>Cutibacterium acnes</i> HL043PA2]	45,4128	16,51	436
327449935	MoeA N-terminal region (domain I and II) [ <i>Cutibacterium acnes</i> HL043PA2]	45,5819	6,9	435
327442867	NADH dehydrogenase subunit D [ <i>Cutibacterium acnes</i> HL043PA2]	47,9817	8,31	433
327445779	hypothetical protein HMPREF9571_01515 [ <i>Cutibacterium acnes</i> HL043PA2]	49,0619	20,37	432

327448378	adenylosuccinate synthase [ <i>Cutibacterium acnes</i> HL043PA2]	47,33	20,83	432
327446554	nucleotide sugar dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	46,8528	15,05	432
327444291	hypothetical protein HMPREF9571_02424 [ <i>Cutibacterium acnes</i> HL043PA2]	47,0956	5,13	429
327445165	aminopeptidase I zinc metalloprotease (M18) [ <i>Cutibacterium acnes</i> HL043PA2]	45,9351	5,14	428
327449197	phosphoribosylaminoimidazole carboxylase, ATPase subunit [ <i>Cutibacterium acnes</i> HL043PA2]	45,8699	6,34	426
327449991	phosphopyruvate hydratase [ <i>Cutibacterium acnes</i> HL043PA2]	45,514	46,24	426
327449117	serine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	46,5027	9,43	424
327444244	FeS assembly protein SufD [ <i>Cutibacterium acnes</i> HL043PA2]	45,8413	17,22	424
327447713	hypothetical protein HMPREF9571_00996 [ <i>Cutibacterium acnes</i> HL043PA2]	43,2017	46,23	424
327449848	sirohydrochlorin cobaltochelataze [ <i>Cutibacterium acnes</i> HL043PA2]	45,4854	4,74	422
327448438	phosphoribosylaminoimidazolesuccinocarboxamide synthase [ <i>Cutibacterium acnes</i> HL043PA2]	46,4004	15,24	420
327446199	cell division protein FtsZ [ <i>Cutibacterium acnes</i> HL043PA2]	42,8086	14,87	417
327446603	periplasmic binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	45,0928	7,45	416
327446722	DNA polymerase III, beta subunit [ <i>Cutibacterium acnes</i> HL043PA2]	44,0732	16,18	414
327444241	cysteine desulfurase, SufS subfamily [ <i>Cutibacterium acnes</i> HL043PA2]	44,4723	8,23	413
327445752	phosphopantothenoylcysteine decarboxylase/phosphopantothenate--cysteine ligase [ <i>Cutibacterium acnes</i> HL043PA2]	42,7904	4,39	410

327445093	glucose-1-phosphate adenylyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	44,7392	5,64	408
327444204	1-deoxy-D-xylulose 5-phosphate reductoisomerase [ <i>Cutibacterium acnes</i> HL043PA2]	42,3727	10,07	407
327445166	putative flagellar protein FliS [ <i>Cutibacterium acnes</i> HL043PA2]	44,2509	8,62	406
327446041	hypothetical protein HMPREF9571_01783 [ <i>Cutibacterium acnes</i> HL043PA2]	43,1674	23,95	405
327445862	Phosphofructokinase [ <i>Cutibacterium acnes</i> HL043PA2]	43,4902	32,43	404
327446143	phosphoglycerate kinase [ <i>Cutibacterium acnes</i> HL043PA2]	42,1912	47,01	402
327449390	translation elongation factor Tu [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	44,1124	37,78	397
327444122	signal recognition particle-docking protein FtsY [ <i>Cutibacterium acnes</i> HL043PA2]	41,2367	12,37	396
327445985	ATPase family associated with various cellular activities (AAA) [ <i>Cutibacterium acnes</i> HL043PA2]	42,687	13,16	395
327449826	glycine C-acetyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	42,8816	14,72	394
327446048	putative chaperone protein DnaJ [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	41,8327	12,76	392
327449137	succinate-CoA ligase, beta subunit [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	41,3311	37,85	391
327447636	FHA domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	41,1582	8,95	391
327451178	TIGR00730 family protein [ <i>Cutibacterium acnes</i> HL043PA2]	42,206	4,11	389
327443362	NlpC/P60 family protein [ <i>Cutibacterium acnes</i> HL043PA2]	40,3889	24,48	388
327444119	amidohydrolase family protein [ <i>Cutibacterium</i> <i>acnes</i> HL043PA2]	41,9412	8,27	387
327445088	hypothetical protein HMPREF9571_02074	40,7534	8,55	386

	[ <i>Cutibacterium acnes</i> HL043PA2]			
327443707	tryptophan--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	41,4214	6,77	384
327449759	phosphate ABC transporter, phosphate-binding protein PstS [ <i>Cutibacterium acnes</i> HL043PA2]	39,5228	31,33	383
327449121	acyl-CoA dehydrogenase, C-terminal domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	41,2717	5,24	382
327448424	chaperone protein DnaJ [ <i>Cutibacterium acnes</i> HL043PA2]	40,5873	8,16	380
327445154	Amidinotransferase [ <i>Cutibacterium acnes</i> HL043PA2]	41,6477	8,73	378
327445101	Amidinotransferase [ <i>Cutibacterium acnes</i> HL043PA2]	43,329	28,99	376
327446955	UDP-N-acetylenolpyruvoylglucosamine reductase domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	39,863	5,59	376
327449922	putative N-acetylglucosamine-6-phosphate deacetylase [ <i>Cutibacterium acnes</i> HL043PA2]	39,1119	10,64	376
327445144	nucleotide sugar dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	41,4731	6,12	376
327446202	putative undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	38,2132	6,72	372
327443908	peptide chain release factor 2 [ <i>Cutibacterium acnes</i> HL043PA2]	41,4429	17,25	371
327446217	aminomethyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	39,546	4,85	371
327445351	hypothetical protein HMPREF9571_01989 [ <i>Cutibacterium acnes</i> HL043PA2]	39,0608	39,08	371
327446565	DegT/DnrJ/EryC1/StrS aminotransferase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	39,2338	21,35	370
327444578	alanine dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	38,1094	14,05	370

327444365	phenylalanine--tRNA ligase, alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	40,1231	5,96	369
327444248	ABC transporter, solute-binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	39,7604	5,98	368
327447529	IMP dehydrogenase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	39,3409	40,87	367
327445105	succinyl-diaminopimelate transaminase [ <i>Cutibacterium acnes</i> HL043PA2]	39,0519	4,9	367
327449727	hypothetical protein HMPREF9571_00293 [ <i>Cutibacterium acnes</i> HL043PA2]	40,2009	10,11	366
327449816	ABC transporter, ATP-binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	39,6844	9,84	366
327447700	oxidoreductase, FAD/FMN-binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	39,0556	15,85	366
327443902	D-ala D-ala ligase N-terminal domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	39,2359	5,21	365
327449152	malate dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	38,7348	33,52	364
327446729	FemAB family protein [ <i>Cutibacterium acnes</i> HL043PA2]	40,7178	9,64	363
327443892	branched-chain-amino-acid transaminase [ <i>Cutibacterium acnes</i> HL043PA2]	39,8763	31,96	363
327446194	DivIVA domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	40,1145	38,5	361
327446942	thiamine pyrophosphate enzyme, C-terminal TPP binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	38,4973	9,97	361
327445681	threonine synthase [ <i>Cutibacterium acnes</i> HL043PA2]	37,4223	6,15	358
327446550	UDP-N-acetylglucosamine 2-epimerase [ <i>Cutibacterium acnes</i> HL043PA2]	38,4964	7,28	357
327443873	peptidyl-prolyl cis-trans isomerase, FKBP-type [ <i>Cutibacterium acnes</i> HL043PA2]	36,8285	10,64	357

327445771	hypothetical protein HMPREF9571_01507 [ <i>Cutibacterium acnes</i> HL043PA2]	40,1423	48,31	356
327446649	UDP-glucose 4-epimerase [ <i>Cutibacterium acnes</i> HL043PA2]	38,5445	11,33	353
327447668	pyruvate dehydrogenase E1 component, alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	38,2386	6,27	351
327445153	ornithine carbamoyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	38,5804	23,14	350
327446776	thioredoxin-disulfide reductase [ <i>Cutibacterium acnes</i> HL043PA2]	37,2393	10,57	350
327445138	LAO/AO transport system ATPase [ <i>Cutibacterium acnes</i> HL043PA2]	37,2082	19,83	348
327445947	RecA protein [ <i>Cutibacterium acnes</i> HL043PA2]	37,1302	36,21	348
327448421	GroES-like protein [ <i>Cutibacterium acnes</i> HL043PA2]	36,9161	8,07	347
327449734	aspartate-semialdehyde dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	35,9806	22,77	347
327449480	putative glycoprotease GCP [ <i>Cutibacterium acnes</i> HL043PA2]	35,9716	16,43	347
327446664	ABC transporter, ATP-binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	36,8586	13,37	344
327446832	putative streptolysin associated protein SagB [ <i>Cutibacterium acnes</i> HL043PA2]	36,8756	20,7	343
327443887	ketol-acid reductoisomerase [ <i>Cutibacterium acnes</i> HL043PA2]	37,3436	6,43	342
327445684	putative transcription termination factor Rho [ <i>Cutibacterium acnes</i> HL043PA2]	38,3643	14,91	342
327446730	alanine racemase domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	37,1985	5,56	342
327448397	fructose-bisphosphate aldolase, class II [ <i>Cutibacterium acnes</i> HL043PA2]	36,8194	27,27	341
327444163	riboflavin biosynthesis protein RibF [ <i>Cutibacterium acnes</i> HL043PA2]	36,6851	7,65	340



327446049	heat-inducible transcription repressor HrcA [ <i>Cutibacterium acnes</i> HL043PA2]	36,3582	9,14	339
327449434	DNA-directed RNA polymerase, alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	36,8267	36,39	338
327449834	oxidoreductase, zinc-binding dehydrogenase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	36,165	9,17	338
327450261	hypothetical protein HMPREF9571_00242 [ <i>Cutibacterium acnes</i> HL043PA2]	36,2868	5,04	337
327446033	prolyl aminopeptidase [ <i>Cutibacterium acnes</i> HL043PA2]	37,7714	17,01	335
327449699	hypothetical protein HMPREF9571_00265 [ <i>Cutibacterium acnes</i> HL043PA2]	37,4598	7,16	335
327446144	glyceraldehyde-3-phosphate dehydrogenase, type I [ <i>Cutibacterium acnes</i> HL043PA2]	35,9276	73,13	335
327451095	hypothetical protein HMPREF9571_00034, partial [ <i>Cutibacterium acnes</i> HL043PA2]	34,3863	34,63	335
327447667	Transketolase, pyridine binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	36,3015	21,26	334
327443377	dihydroxyacetone kinase, DhaK subunit [ <i>Cutibacterium acnes</i> HL043PA2]	34,6556	10,21	333
327445977	beta-ketoacyl-acyl-carrier-protein synthase III [ <i>Cutibacterium acnes</i> HL043PA2]	35,2539	8,13	332
327449896	AP endonuclease, family 2 [ <i>Cutibacterium acnes</i> HL043PA2]	36,5881	14,46	332
327444359	tyrosine--tRNA ligase [ <i>Cutibacterium acnes</i> HL043PA2]	36,7827	5,44	331
327446564	oxidoreductase, NAD-binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	35,5402	26,67	330
327446833	hypothetical protein HMPREF9571_01087 [ <i>Cutibacterium acnes</i> HL043PA2]	35,7231	10,06	328
327450410	putative dihydroorotate dehydrogenase 2 [ <i>Cutibacterium acnes</i> HL043PA2]	34,6635	32,42	327
327442879	polyprenyl synthetase [ <i>Cutibacterium acnes</i>	34,6627	8,95	324



	HL043PA2]			
327444188	transcription termination factor NusA [ <i>Cutibacterium acnes</i> HL043PA2]	35,7166	4,64	323
327448382	dihydrodipicolinate synthetase family [ <i>Cutibacterium acnes</i> HL043PA2]	34,4606	6,81	323
327445148	oxidoreductase, aldo/keto reductase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	34,8244	17,08	322
327446078	L-lactate dehydrogenase [ <i>Cutibacterium acnes</i> HL043PA2]	33,9924	6,83	322
327443371	lactate/malate dehydrogenase, NAD binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	33,6428	15,89	321
327446213	ATPase family associated with various cellular activities (AAA) [ <i>Cutibacterium acnes</i> HL043PA2]	34,395	9,12	318
327445829	methionine synthase, vitamin-B12 independent [ <i>Cutibacterium acnes</i> HL043PA2]	33,5973	5,71	315
327446780	putative sugar-binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	33,5603	6,98	315
327448409	putative fructose-bisphosphate aldolase class-I [ <i>Cutibacterium acnes</i> HL043PA2]	34,724	37,26	314
327445964	aspartate carbamoyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	34,7149	28,66	314
327445698	ATP synthase F1, gamma subunit [ <i>Cutibacterium acnes</i> HL043PA2]	34,5417	23,89	314
327445843	thioredoxin [ <i>Cutibacterium acnes</i> HL043PA2]	33,1149	5,43	313
327449719	porphobilinogen synthase [ <i>Cutibacterium acnes</i> HL043PA2]	33,4195	23,47	311
327446950	enoyl-CoA hydratase/isomerase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	33,1245	7,72	311
327449976	ribose-phosphate diphosphokinase [ <i>Cutibacterium acnes</i> HL043PA2]	33,9934	9,35	310
327449994	Ppx/GppA phosphatase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	32,7367	6,45	310
327445807	N-acetylmuramic acid 6-phosphate etherase	31,2779	6,58	304

	[ <i>Cutibacterium acnes</i> HL043PA2]			
327446958	transcription termination/antitermination factor NusG [ <i>Cutibacterium acnes</i> HL043PA2]	33,4752	9,9	303
327445040	Tat pathway signal sequence domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	32,5854	8,91	303
327449970	hydrolase, TatD family [ <i>Cutibacterium acnes</i> HL043PA2]	32,3613	8,94	302
327445739	hypothetical protein HMPREF9571_01475 [ <i>Cutibacterium acnes</i> HL043PA2]	33,2098	10,96	301
327446059	naphthoate synthase [ <i>Cutibacterium acnes</i> HL043PA2]	33,1334	8,31	301
327449401	50S ribosomal protein L4 [ <i>Cutibacterium acnes</i> HL043PA2]	32,2785	43,52	301
327445152	carbamate kinase [ <i>Cutibacterium acnes</i> HL043PA2]	31,9773	8,31	301
327449477	hydrolase, P-loop family [ <i>Cutibacterium acnes</i> HL043PA2]	30,7598	9,09	297
327449138	succinate-CoA ligase, alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	30,5618	48,15	297
327446684	putative histidinol-phosphate transaminase [ <i>Cutibacterium acnes</i> HL043PA2]	32,0123	13,22	295
327448374	putative alkyl hydroperoxide reductase, F subunit, partial [ <i>Cutibacterium acnes</i> HL043PA2]	31,1971	6,78	295
327445091	dihydrodipicolinate synthase [ <i>Cutibacterium acnes</i> HL043PA2]	30,6707	7,17	293
327451296	Tat pathway signal sequence domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	30,5829	8,53	293
327449890	myo-inositol catabolism protein IolB [ <i>Cutibacterium acnes</i> HL043PA2]	31,7904	22,76	290
327445976	hypothetical protein HMPREF9571_01716 [ <i>Cutibacterium acnes</i> HL043PA2]	30,0844	30,34	290
327445184	oxidoreductase, aldo/keto reductase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	31,525	11,85	287

327449149	tetrahydrofolate dehydrogenase/cyclohydrolase, NAD(P)-binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	30,2778	39,37	287
327443795	hypothetical protein HMPREF9571_02536 [ <i>Cutibacterium acnes</i> HL043PA2]	30,3937	30,18	285
327444372	ATP phosphoribosyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	30,5918	15,14	284
327445930	universal stress family protein [ <i>Cutibacterium acnes</i> HL043PA2]	29,2281	15,14	284
327444215	ribosomal protein S2 [ <i>Cutibacterium acnes</i> HL043PA2]	31,5164	32,51	283
327449153	putative formyltetrahydrofolate deformylase [ <i>Cutibacterium acnes</i> HL043PA2]	31,1939	10,25	283
327445745	hypothetical protein HMPREF9571_01481 [ <i>Cutibacterium acnes</i> HL043PA2]	29,8242	6,05	281
327444299	acyl-CoA thioester hydrolase, YbgC/YbaW family [ <i>Cutibacterium acnes</i> HL043PA2]	30,8817	7,47	281
327449428	methionine aminopeptidase, type I [ <i>Cutibacterium acnes</i> HL043PA2]	29,959	7,53	279
327445997	pyridoxal 5'-phosphate synthase, synthase subunit Pdx1 [ <i>Cutibacterium acnes</i> HL043PA2]	29,1149	68,46	279
327449403	ribosomal protein L2 [ <i>Cutibacterium acnes</i> HL043PA2]	30,2282	17,63	278
327445737	proteasome, beta subunit [ <i>Cutibacterium acnes</i> HL043PA2]	29,5598	8,63	278
327446080	putative hydroxyethylthiazole kinase [ <i>Cutibacterium acnes</i> HL043PA2]	28,3904	12,64	277
327442866	NADH dehydrogenase, C subunit [ <i>Cutibacterium acnes</i> HL043PA2]	30,569	11,68	274
327445939	diaminopimelate epimerase [ <i>Cutibacterium acnes</i> HL043PA2]	29,7795	8,79	273
327447715	oxidoreductase, aldo/keto reductase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	29,721	12,09	273

327445719	glycosyltransferase, group 2 family protein [ <i>Cutibacterium acnes</i> HL043PA2]	30,1724	10,29	272
327443369	putative HAD hydrolase, TIGR01457 family [ <i>Cutibacterium acnes</i> HL043PA2]	29,732	12,87	272
327449116	hypothetical protein HMPREF9571_00834 [ <i>Cutibacterium acnes</i> HL043PA2]	29,7289	20,59	272
327451135	hypothetical protein HMPREF9571_00078 [ <i>Cutibacterium acnes</i> HL043PA2]	29,1008	15,13	271
327444158	hypothetical protein HMPREF9571_02289 [ <i>Cutibacterium acnes</i> HL043PA2]	29,2495	8,86	271
327444214	translation elongation factor Ts [ <i>Cutibacterium acnes</i> HL043PA2]	28,4705	31,85	270
327449173	putative purine nucleotide phosphorylase [ <i>Cutibacterium acnes</i> HL043PA2]	27,6565	30	270
327450249	DoxX family protein [ <i>Cutibacterium acnes</i> HL043PA2]	29,4094	14,81	270
327449406	ribosomal protein S3 [ <i>Cutibacterium acnes</i> HL043PA2]	29,6556	34,57	269
327449069	aminotransferase, class IV [ <i>Cutibacterium acnes</i> HL043PA2]	28,8386	11,52	269
327446535	CobQ/CobB/MinD/ParA nucleotide binding domain protein, partial [ <i>Cutibacterium acnes</i> HL043PA2]	27,8867	7,84	268
327445028	PspA/IM30 family protein [ <i>Cutibacterium acnes</i> HL043PA2]	29,5078	46,44	267
327449184	putative phosphomethylpyrimidine kinase [ <i>Cutibacterium acnes</i> HL043PA2]	27,9133	23,22	267
327446560	oxidoreductase, short chain dehydrogenase/reductase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	28,5475	38,35	266
327445789	DNA-binding regulatory protein, YebC/PmpR family [ <i>Cutibacterium acnes</i> HL043PA2]	28,7785	7,58	264
327449745	oxidoreductase, short chain	28,1674	16,67	264

	dehydrogenase/reductase family protein [ <i>Cutibacterium acnes</i> HL043PA2]			
327445696	ATP synthase F1, delta subunit [ <i>Cutibacterium acnes</i> HL043PA2]	28,4299	26,62	263
327442868	NADH dehydrogenase subunit E [ <i>Cutibacterium acnes</i> HL043PA2]	28,4199	19,92	261
327444226	ABC transporter, ATP-binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	28,0096	17,24	261
327446142	triose-phosphate isomerase [ <i>Cutibacterium acnes</i> HL043PA2]	27,4409	22,01	259
327449756	phosphate ABC transporter, ATP-binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	28,3666	8,14	258
327449863	uroporphyrinogen-III C-methyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	26,7818	31,78	258
327444242	FeS assembly ATPase SufC [ <i>Cutibacterium acnes</i> HL043PA2]	27,9633	43,97	257
327444112	succinate dehydrogenase/fumarate reductase iron-sulfur subunit [ <i>Cutibacterium acnes</i> HL043PA2]	27,5455	14,4	257
327444228	putative enoyl-(acyl carrier protein) reductase [ <i>Cutibacterium acnes</i> HL043PA2]	27,214	13,62	257
327445081	putative zinc ribbon domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	28,6266	9,38	256
327449702	LamB/YcsF family protein [ <i>Cutibacterium acnes</i> HL043PA2]	26,5104	15,63	256
327449227	tRNA nucleotidyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	27,1039	14,17	254
327449867	hypothetical protein HMPREF9571_00433 [ <i>Cutibacterium acnes</i> HL043PA2]	28,3866	10,24	254
327449784	phosphoglycerate mutase 1 family [ <i>Cutibacterium acnes</i> HL043PA2]	27,9732	34,54	249
327446150	phosphoglycerate mutase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	26,9457	7,66	248
327444159	dihydrodipicolinate reductase [ <i>Cutibacterium acnes</i> HL043PA2]	25,928	13,82	246

	HL043PA2]			
327443872	pseudouridylate synthase [ <i>Cutibacterium acnes</i> HL043PA2]	27,3936	16,33	245
327449769	oxidoreductase, short chain dehydrogenase/reductase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	25,701	14,69	245
327445900	hypothetical protein HMPREF9571_01640 [ <i>Cutibacterium acnes</i> HL043PA2]	26,91	46,72	244
327447632	response regulator receiver domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	27,3162	13,58	243
327450482	cyclic nucleotide-binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	26,2065	11,93	243
327449720	uroporphyrinogen-III synthase [ <i>Cutibacterium acnes</i> HL043PA2]	25,1626	10,37	241
327449923	iron dependent repressor DNA binding domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	26,2862	10	240
327445326	FCD domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	26,0642	20,08	239
327443896	transcriptional regulator, IclR family, C-terminal domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	24,9494	14,64	239
327444201	TENA/THI-4 family protein [ <i>Cutibacterium acnes</i> HL043PA2]	26,6043	10,46	239
327444213	UMP kinase [ <i>Cutibacterium acnes</i> HL043PA2]	25,6977	36,13	238
327445958	orotidine 5'-phosphate decarboxylase [ <i>Cutibacterium acnes</i> HL043PA2]	24,3475	16,53	236
327446551	glycosyltransferase, group 1 family protein [ <i>Cutibacterium acnes</i> HL043PA2]	25,1218	11,54	234
327445671	RNA polymerase sigma-70 factor [ <i>Cutibacterium acnes</i> HL043PA2]	26,3122	11,11	234
327446960	ribosomal protein L1 [ <i>Cutibacterium acnes</i> HL043PA2]	24,7229	43,1	232
327445157	DJ-1/PfpI family protein [ <i>Cutibacterium acnes</i> HL043PA2]	24,4872	10,92	229

327448381	putative N-acetylmannosamine-6-phosphate epimerase [ <i>Cutibacterium acnes</i> HL043PA2]	24,0112	31,44	229
327449423	glycerophosphodiester phosphodiesterase family protein [ <i>Cutibacterium acnes</i> HL043PA2]	25,1787	13,16	228
327443909	cell division ATP-binding protein FtsE [ <i>Cutibacterium acnes</i> HL043PA2]	25,5515	8,77	228
327446163	periplasmic binding protein [ <i>Cutibacterium acnes</i> HL043PA2]	24,6392	12,78	227
327445736	proteasome, alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	24,2974	7,96	226
327450420	hypothetical protein HMPREF9571_00142 [ <i>Cutibacterium acnes</i> HL043PA2]	23,9674	7,08	226
327449779	response regulator receiver domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	25,1562	12,83	226
327445897	TrkA N-terminal domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	24,734	22,22	225
327449400	50S ribosomal protein L3 [ <i>Cutibacterium acnes</i> HL043PA2]	23,4984	28,25	223
327446018	response regulator receiver domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	23,9958	8,07	223
327445744	ribulose-phosphate 3-epimerase [ <i>Cutibacterium acnes</i> HL043PA2]	24,1126	16,14	223
327449781	phosphate transport system regulatory protein PhoU [ <i>Cutibacterium acnes</i> HL043PA2]	24,4103	18,47	222
327448425	co-chaperone GrpE [ <i>Cutibacterium acnes</i> HL043PA2]	24,3015	12,22	221
327445702	ATP:cob(I)alamin adenosyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	24,0792	15,84	221
327445908	hypothetical protein HMPREF9571_01648 [ <i>Cutibacterium acnes</i> HL043PA2]	23,7866	12,27	220
327445296	hypothetical protein HMPREF9571_02000 [ <i>Cutibacterium acnes</i> HL043PA2]	23,1003	26,15	218
327443376	dihydroxyacetone kinase, L subunit [ <i>Cutibacterium</i>	22,1231	32,57	218



	<i>acnes</i> HL043PA2]			
327445092	O-methyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	23,3181	11,06	217
327445957	orotate phosphoribosyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	23,0188	13,89	216
327446957	preprotein translocase, SecE subunit [ <i>Cutibacterium acnes</i> HL043PA2]	23,5371	16,2	216
327446187	response regulator receiver domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	23,8211	37,67	215
327449070	transcriptional regulator, TetR family [ <i>Cutibacterium acnes</i> HL043PA2]	23,3531	26,05	215
327445339	hypothetical protein HMPREF9571_01977 [ <i>Cutibacterium acnes</i> HL043PA2]	23,2977	8,37	215
327449417	ribosomal protein S5 [ <i>Cutibacterium acnes</i> HL043PA2]	22,5519	30,7	215
327444369	translation initiation factor IF-3 [ <i>Cutibacterium acnes</i> HL043PA2]	24,0418	27,23	213
327443802	ribosomal subunit interface protein [ <i>Cutibacterium acnes</i> HL043PA2]	23,7463	37,74	212
327445822	TIGR03085 family protein [ <i>Cutibacterium acnes</i> HL043PA2]	23,1272	13,74	211
327449412	ribosomal protein L5 [ <i>Cutibacterium acnes</i> HL043PA2]	23,8435	35,24	210
327445670	DoxX family protein [ <i>Cutibacterium acnes</i> HL043PA2]	21,9569	29,52	210
327451126	uracil phosphoribosyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	22,6387	17,79	208
327451297	hypothetical protein HMPREF9571_00027 [ <i>Cutibacterium acnes</i> HL043PA2]	21,9161	10,14	207
327449144	phosphoribosylglycinamide formyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	22,2486	11,11	207
327449980	ribosomal protein L25, Ctc-form [ <i>Cutibacterium acnes</i> HL043PA2]	22,3085	7,8	205



327446566	bacterial transferase hexapeptide repeat protein [ <i>Cutibacterium acnes</i> HL043PA2]	21,7039	31,71	205
327449442	superoxide dismutase [ <i>Cutibacterium acnes</i> HL043PA2]	23,3234	8,29	205
327446961	ribosomal protein L10 [ <i>Cutibacterium acnes</i> HL043PA2]	20,9161	50,49	204
327449141	riboflavin synthase, alpha subunit [ <i>Cutibacterium acnes</i> HL043PA2]	21,5371	15,69	204
327446728	R3H domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	22,2115	8,87	203
327444311	oligoribonuclease [ <i>Cutibacterium acnes</i> HL043PA2]	22,5422	23,27	202
327449433	ribosomal protein S4 [ <i>Cutibacterium acnes</i> HL043PA2]	23,1714	35,82	201
327442872	NADH-quinone oxidoreductase, chain I [ <i>Cutibacterium acnes</i> HL043PA2]	22,3223	17,17	198
327445981	pyridoxal phosphate enzyme, YggS family [ <i>Cutibacterium acnes</i> HL043PA2]	21,785	11,11	198
327445869	FHA domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	20,4459	22,4	192
327450463	transcriptional regulator, PadR family [ <i>Cutibacterium acnes</i> HL043PA2]	21,441	13,61	191
327445965	pyrimidine operon regulatory protein/uracil phosphoribosyltransferase PyrR [ <i>Cutibacterium acnes</i> HL043PA2]	20,8941	46,6	191
327445757	translation elongation factor P [ <i>Cutibacterium acnes</i> HL043PA2]	21,1367	8,47	189
327449427	adenylate kinase [ <i>Cutibacterium acnes</i> HL043PA2]	20,8857	35,45	189
327448439	peroxiredoxin [ <i>Cutibacterium acnes</i> HL043PA2]	20,8042	35,29	187
327444212	ribosome recycling factor [ <i>Cutibacterium acnes</i> HL043PA2]	20,9168	38,04	184
327450262	inorganic diphosphatase [ <i>Cutibacterium acnes</i> HL043PA2]	20,7478	10,33	184

327445695	ATP synthase F0, B subunit [ <i>Cutibacterium acnes</i> HL043PA2]	20,1494	11,96	184
327449105	single-strand binding family protein [ <i>Cutibacterium acnes</i> HL043PA2]	19,3752	16,85	184
327449415	ribosomal protein L6 [ <i>Cutibacterium acnes</i> HL043PA2]	19,8247	31,67	180
327449435	ribosomal protein L17 [ <i>Cutibacterium acnes</i> HL043PA2]	19,1911	20,11	179
327444116	16S rRNA processing protein RimM [ <i>Cutibacterium acnes</i> HL043PA2]	19,428	13,97	179
327451159	intracellular protease, PfpI family [ <i>Cutibacterium acnes</i> HL043PA2]	19,0128	16,76	179
327444189	hypothetical protein HMPREF9571_02320 [ <i>Cutibacterium acnes</i> HL043PA2]	19,5229	9,55	178
327450229	hypothetical protein HMPREF9571_00209 [ <i>Cutibacterium acnes</i> HL043PA2]	18,1967	19,1	178
327445782	adenine phosphoribosyltransferase [ <i>Cutibacterium acnes</i> HL043PA2]	18,3547	20,9	177
327447720	ferritin-like protein [ <i>Cutibacterium acnes</i> HL043PA2]	19,0296	18,18	176
327450278	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase [ <i>Cutibacterium acnes</i> HL043PA2]	19,0767	16,57	175
327448385	hypothetical protein HMPREF9571_00868 [ <i>Cutibacterium acnes</i> HL043PA2]	19,1431	13,14	175
327446588	hypothetical protein HMPREF9571_01154 [ <i>Cutibacterium acnes</i> HL043PA2]	19,9241	20,69	174
327446831	hypothetical protein HMPREF9571_01086 [ <i>Cutibacterium acnes</i> HL043PA2]	19,7591	9,77	174
327449457	ribosomal protein S9 [ <i>Cutibacterium acnes</i> HL043PA2]	18,5878	20,81	173
327446196	hypothetical protein HMPREF9571_01940 [ <i>Cutibacterium acnes</i> HL043PA2]	19,4377	13,95	172

327449110	ferritin-like protein [ <i>Cutibacterium acnes</i> HL043PA2]	19,0304	36,05	172
327449808	hypothetical protein HMPREF9571_00374 [ <i>Cutibacterium acnes</i> HL043PA2]	18,5022	18,02	172
327445149	ACT domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	18,3715	13,95	172
327449988	hypothetical protein HMPREF9571_00558 [ <i>Cutibacterium acnes</i> HL043PA2]	17,9651	11,7	171
327445350	peptidyl-prolyl cis-trans isomerase, cyclophilin-type [ <i>Cutibacterium acnes</i> HL043PA2]	18,3522	22,35	170
327445178	prokaryotic transcription elongation factor, GreA/GreB domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	18,3008	12,5	168
327444240	SUF system FeS assembly protein, NifU family [ <i>Cutibacterium acnes</i> HL043PA2]	18,2898	22,89	166
327449143	6,7-dimethyl-8-ribityllumazine synthase [ <i>Cutibacterium acnes</i> HL043PA2]	16,7725	10,84	166
327445050	PTS system, glucose subfamily, IIA component [ <i>Cutibacterium acnes</i> HL043PA2]	16,8165	15,24	164
327449777	CarD-like protein [ <i>Cutibacterium acnes</i> HL043PA2]	18,1987	30,43	161
327446193	C4-type zinc finger protein, DksA/TraR family [ <i>Cutibacterium acnes</i> HL043PA2]	17,2182	9,43	159
327451171	hypothetical protein HMPREF9571_00115, partial [ <i>Cutibacterium acnes</i> HL043PA2]	16,6993	14,56	158
327444290	ribose 5-phosphate isomerase [ <i>Cutibacterium acnes</i> HL043PA2]	17,2045	17,2	157
327448423	transcriptional regulator, MerR family [ <i>Cutibacterium acnes</i> HL043PA2]	17,249	10,46	153
327449405	ribosomal protein L22 [ <i>Cutibacterium acnes</i> HL043PA2]	16,699	30,07	153
327449100	phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2 [ <i>Cutibacterium</i>	15,9764	17,65	153

	<i>acnes</i> HL043PA2]			
327445848	methylmalonyl-CoA epimerase [ <i>Cutibacterium acnes</i> HL043PA2]	16,7934	25,66	152
327445329	Hsp20/alpha crystallin family protein [ <i>Cutibacterium acnes</i> HL043PA2]	16,6995	23,68	152
327445969	hypothetical protein HMPREF9571_01709 [ <i>Cutibacterium acnes</i> HL043PA2]	16,426	19,21	151
327450481	endoribonuclease L-PSP [ <i>Cutibacterium acnes</i> HL043PA2]	14,9469	23,84	151
327449107	ribosomal protein L9 [ <i>Cutibacterium acnes</i> HL043PA2]	16,108	22,15	149
327444118	ribosomal protein S16 [ <i>Cutibacterium acnes</i> HL043PA2]	15,9462	52,38	147
327445700	ATP synthase F1, epsilon subunit [ <i>Cutibacterium acnes</i> HL043PA2]	16,0202	15,07	146
327449925	thioredoxin [ <i>Cutibacterium acnes</i> HL043PA2]	16,0921	23,45	145
327446772	OsmC-like protein [ <i>Cutibacterium acnes</i> HL043PA2]	15,3427	31,03	145
327444580	ribosomal protein S7 [ <i>Cutibacterium acnes</i> HL043PA2]	16,2586	29,17	144
327444180	ribosome-binding factor A [ <i>Cutibacterium acnes</i> HL043PA2]	15,592	36,36	143
327446959	ribosomal protein L11 [ <i>Cutibacterium acnes</i> HL043PA2]	15,125	16,78	143
327445159	peroxiredoxin, Ohr subfamily [ <i>Cutibacterium acnes</i> HL043PA2]	14,7333	28,17	142
327449945	TOBE domain protein [ <i>Cutibacterium acnes</i> HL043PA2]	15,3212	20,42	142
327446137	nucleoside diphosphate kinase [ <i>Cutibacterium acnes</i> HL043PA2]	15,3429	19,57	138
327446542	hypothetical protein HMPREF9571_01107 [ <i>Cutibacterium acnes</i> HL043PA2]	15,2666	18,98	137
327446043	DoxX family protein [ <i>Cutibacterium acnes</i> HL043PA2]	13,7245	19,55	133

	HL043PA2]			
327446962	ribosomal protein L7/L12 [ <i>Cutibacterium acnes</i> HL043PA2]	13,6932	28,46	130
327446536	single-strand binding family protein [ <i>Cutibacterium acnes</i> HL043PA2]	14,1052	17,97	128
327449416	ribosomal protein L18 [ <i>Cutibacterium acnes</i> HL043PA2]	13,6924	19,69	127
327446705	RbsD/FucU transport family protein [ <i>Cutibacterium acnes</i> HL043PA2]	13,8192	18,9	127
327449431	30S ribosomal protein S13 [ <i>Cutibacterium acnes</i> HL043PA2]	13,9878	25,2	123
327444367	ribosomal protein L20 [ <i>Cutibacterium acnes</i> HL043PA2]	13,9514	21,14	123
327446218	glycine cleavage system H protein [ <i>Cutibacterium acnes</i> HL043PA2]	12,9532	16,26	123
327449410	ribosomal protein L14 [ <i>Cutibacterium acnes</i> HL043PA2]	13,4704	24,59	122
327449411	ribosomal protein L24 [ <i>Cutibacterium acnes</i> HL043PA2]	13,4724	18,03	122
327448389	methylmalonyl-CoA carboxyltransferase 1.3S subunit [ <i>Cutibacterium acnes</i> HL043PA2]	11,6191	21,67	120
327447699	hypothetical protein HMPREF9571_00982 [ <i>Cutibacterium acnes</i> HL043PA2]	13,3495	27,73	119
327444351	ribosomal protein L19 [ <i>Cutibacterium acnes</i> HL043PA2]	13,0711	28,21	117
327445025	iron-sulfur cluster assembly accessory protein [ <i>Cutibacterium acnes</i> HL043PA2]	12,4641	13,68	117
327445718	hypothetical protein HMPREF9571_01453 [ <i>Cutibacterium acnes</i> HL043PA2]	12,7241	18,18	110
327450282	hypothetical protein HMPREF9571_00263 [ <i>Cutibacterium acnes</i> HL043PA2]	11,7749	20	105
327450413	hypothetical protein HMPREF9571_00135 [ <i>Cutibacterium acnes</i> HL043PA2]	11,2758	23,81	105

327449399	ribosomal protein S10 [ <i>Cutibacterium acnes</i> HL043PA2]	11,6723	26,21	103
327451377	Ribbon-helix-helix protein, CopG family [ <i>Cutibacterium acnes</i> HL043PA2]	10,9405	31,07	103
327449402	ribosomal protein L23 [ <i>Cutibacterium acnes</i> HL043PA2]	11,3232	39,22	102
327445904	hypothetical protein HMPREF9571_01644 [ <i>Cutibacterium acnes</i> HL043PA2]	11,0151	15,31	98
327449489	chaperonin GroS [ <i>Cutibacterium acnes</i> HL043PA2]	10,6216	43,88	98
327449104	ribosomal protein S6 [ <i>Cutibacterium acnes</i> HL043PA2]	11,2459	19,79	96
327449404	ribosomal protein S19 [ <i>Cutibacterium acnes</i> HL043PA2]	10,5036	50,54	93
327446843	hypothetical protein HMPREF9571_01097 [ <i>Cutibacterium acnes</i> HL043PA2]	10,24	20,65	92
327449219	DNA-binding protein HB1 [ <i>Cutibacterium acnes</i> HL043PA2]	9,5822	28,57	91
327449409	30S ribosomal protein S17 [ <i>Cutibacterium acnes</i> HL043PA2]	10,4456	22,22	90
327446132	ribosomal protein L27 [ <i>Cutibacterium acnes</i> HL043PA2]	9,51694	52,81	89
327446066	ribosomal protein S20 [ <i>Cutibacterium acnes</i> HL043PA2]	9,69442	14,77	88
327449771	phosphocarrier, HPr family [ <i>Cutibacterium acnes</i> HL043PA2]	9,00556	26,14	88
327444162	ribosomal protein S15 [ <i>Cutibacterium acnes</i> HL043PA2]	10,0734	18,39	87
327445978	acyl carrier protein [ <i>Cutibacterium acnes</i> HL043PA2]	9,0223	30,86	81
327448390	hypothetical protein HMPREF9571_00873 [ <i>Cutibacterium acnes</i> HL043PA2]	8,78664	22,5	80
327449106	ribosomal protein S18 [ <i>Cutibacterium acnes</i> HL043PA2]	8,79208	27,85	79

	HL043PA2]			
327449408	ribosomal protein L29 [ <i>Cutibacterium acnes</i> HL043PA2]	8,87972	45,45	77
327450491	hypothetical protein HMPREF9571_00120, partial [ <i>Cutibacterium acnes</i> HL043PA2]	8,48723	46,75	77
327444368	ribosomal protein L35 [ <i>Cutibacterium acnes</i> HL043PA2]	7,88941	13,89	72
327444383	CsbD-like protein [ <i>Cutibacterium acnes</i> HL043PA2]	7,30578	28,17	71
327445685	ribosomal protein L31 [ <i>Cutibacterium acnes</i> HL043PA2]	7,71278	43,48	69
327445941	hypothetical protein HMPREF9571_01681 [ <i>Cutibacterium acnes</i> HL043PA2]	7,16049	84,85	66

## ***General discussion***



## Discussion

Microbial biofilms, being the main form of life of microorganisms in natural ecotopes and in the human body, are constantly exposed to an almost infinite diversity of environmental factors. These can be physical, chemical or biological. During life and anthropogenic activity, human constantly encounters microbial biofilms and has a direct or indirect influence on them. In the field of health care, humans deal, as a rule, with biofilms of pathogenic, opportunistic and symbiotic microorganisms. Most of the research has been devoted to biofilms of the first two types of microorganisms. Biofilms of pathogenic and conditionally pathogenic microorganisms are the cause of chronic infections (Nozhevnikova et al., 2015) and various complications. Many studies have been focused on the effect of antibiotics on biofilms of pathogenic microorganisms, in particular *C. acnes* (Furustrand Tafin et al., 2012, Crane et al., 2013) and *S. aureus* (Bhattacharya et al., 2015; Schilcher et al. 2016). For example, an inhibitory effect of azithromycin on the growth of *S. aureus* biofilms was shown (Wu et al., 2010, Gui et al., 2014, Majidpour et al., 2017). Many works are also focused on pseudomonades (Gillis & Iglewski, 2004; Phelan et al. al., 2015; Das et al., 2016; Li et al., 2017; Saini et al., 2017) and the effects of antibiotics on their biofilms. Because of the increasing number of strains resistant to antibiotics every year (Magiorakos et al., 2012), and the particular resistance of microorganisms in biofilms (Plakunov et al., 2010), the search for anti-biofilm compounds is becoming increasingly important for fundamental science and medicine. Those compounds are not necessarily antibiotics, but they can influence the growth of biofilms (amplify it in the case of symbiotic microorganisms or suppress them in the case of pathogens). Such molecules are of peculiar interest for cosmetic industry and and some active cosmetic compounds disrupt or stimulate the growth of biofilms (cinnamon oil (Aiemsaard et al., 2011), orange oil (Federman et al., 2016), for instance). Biofilms can be also the target of pharmaceutical compounds and this is including molecules that were not produced originally to act on bacteria such as the anthelmintic drug niclosamide (Zhurina et al., 2017) or 4-hexylresorcinol, an antitumor compound, as well as nutritional supplements and antiseptics (Lee et al., 2013). This type of molecules also includes human body's own products, such as hormones (adrenaline (Tiwari et al., 2018), norepinephrine (Pillai et al., 2018), dopamine, insulin, estrogen (Feraco et al., 2016), etc.) or neuropeptides such as substance P and neuropeptides Y (Augustyniak et al., 2012), which were not initially identified for their effect on bacteria, but may have a regulatory effect on biofilms formation. In this thesis, studies have been carried out on the effect of a number of these compounds on microbial biofilms allowing to suggest common properties and potential new applications. The effects of azithromycin on *P. chlororaphis* 449, niclosamide as a promising antibiotic agent on a

number of skin microorganisms, as well as its combination with azithromycin on *S. aureus*, were tested. Cosmetic compounds, namely PS291® and UTW and natriuretic peptides (ANP and CNP), neuropeptides known for their action on *P. aeruginosa* biofilms, were investigated in regard of their potential effects on biofilms formation by cutaneous bacteria *i.e.* *C. acnes*, *S. aureus* and *S. epidermidis*. As in the environment biofilms are rarely pure, in order to get closer from the reality, binary biofilms of *C. acnes*, *S. aureus* and *S. epidermidis* were produced allowing to test the effect of cosmetic products and natriuretic peptides on these complex biofilms. Finally, a special part of this work focused on the study of the chemical composition of the matrix of an acneic strain of *C. acnes* (RT5) as until now our knowledge of *C. acnes* biofilms composition remained very limited. Data obtained during this thesis are of major interest both for fundamental and applied sciences. Identifying the steps of biofilm formation modulated by exogenous and host molecules is improving our knowledge on bacterial resistance into biofilms and their adaptation of the microenvironment. These information have a potential direct impact on cosmetic and pharmaceutical industry. Data about the composition of *C. acnes* matrix makes also possible to select or develop compounds that should specifically modulate biofilm formation by this microbial species.

*P. chlororaphis* 449 and its genetically modified strains were selected as experimental models for multiple reasons. First, *P. chlororaphis* is a rhizosphere saprotrophic microorganism related to opportunistic pseudomonad that also live on the human skin, such as *P. aeruginosa* and *P. fluorescens* (Mishra et al., 2009; Hesse et al., 2018), which makes it a convenient and safe object for modeling processes occurring in pathogenic microorganisms. Secondly, a large amount of data on the effect of antimicrobial compounds on the growth of *P. aeruginosa* biofilms, in particular, azithromycin, has been accumulated in the literature (Tan et al., 2016; Li et al., 2017; Bahari et al., 2017), but very little work is devoted to *P. chlororaphis*. Thus, the study of the effect of antibiotics on *P. chlororaphis* 449 is relevant in that it shows the general biological character of the phenomenon of antibiotic stimulation of biofilm formation. This study is of great importance for understanding the fundamental processes that occur during the formation of biofilms, and the interaction of microorganisms with unfavorable environmental factors.

The main task of working with *P. chlororaphis* was to study the effect of mutations damaging the regulatory systems of the microorganism on the nature of the effect of azithromycin on planktonic cultures and biofilms of *P. chlororaphis* strains 449 and 66. The antibiotic was used as a biochemical tool (protein synthesis inhibitor interacting with the 50S subunit of the ribosome). In a wild type strain of *P. chlororaphis* 449, biofilms proved to be much more resistant to azithromycin than planktonic cells: at concentrations of this antibiotic

that inhibited the growth of planktonic culture by 50%, the growth of biofilms increased 1.5-1.6 times. The effect of stimulating the formation of biofilms was manifested at concentrations of azithromycin, which did not affect the growth of planktonic culture (0.1 µg / ml), and therefore it is not directly related to the ability of an antibiotic to inhibit protein synthesis. Stimulation of the growth of biofilms in the presence of low concentrations of antibiotic is of great practical importance, since it can lead to serious complications in the chemotherapy of infections in case of non-compliance with the duration of treatment or the reduction of effective doses of the antibiotic. On the other hand, clarifying the mechanism of stimulation can help creating a tool to control the formation of biofilms for the purpose of their biotechnological use (for example, in bioreactors for the production of useful products or for sewage treatment). Therefore, in the present study, the study of the mechanisms of this phenomenon has been given increased attention.

Mutations that do not affect the synthesis of AGL (strains of *ΔrpoS*, *Δphz*) do not significantly affect the sensitivity of the planktonic culture of the altered strain to azithromycin, nor the activating effect of this antibiotic with respect to mutant biofilms. The mutation in the *gacS* gene encoding the GacS sensor kinase GacS of the two-component GacA-GacS regulatory system leading to complete suppression of the synthesis of part of AGL (*ΔgacS* strain), without affecting the sensitivity of planktonic culture to azithromycin, significantly reduced the stimulation of biofilm growth in the presence of this antibiotic. Finally, the introduction of plasmid pME6863, including the cloned N-acylhomoserine lactanase gene, *AiiA* and causing degradation of all types of AHL (strain pME6863), resulted in a complete disappearance of the azithromycin stimulatory effect on biofilm growth. Moreover, in this case the bacterium in biofilm became even more sensitive to azithromycin than in planktonic culture. Then, we can draw the important conclusion from these experiments that azithromycin stimulation of biofilm growth is directly related to the functioning of the global regulation QS-system and the obligatory synthesis of its signal components - AHL.

Stimulation of the formation of biofilms is caused not only by antibiotics, but also by certain oxidizing agents, for example, hydrogen peroxide. With the introduction of the same plasmid pME6863 (the mutant *P. chlororaphis* 4490/pME6863) to a *P. aeruginosa* PAO1 strain in which hydrogen peroxide stimulates the growth of biofilms, the effect of stimulation is completely eliminated (Plyuta et al., 2013). Apparently, in this case, antibiotics and oxidizers are stressors, to which the microorganism reacts by transition to a more stable biofilm type of existence, in which the signal components of the quorum sensing system plays a necessary role.

To answer the question about the biochemical mechanisms involved in the stimulation of *P. chlororaphis* biofilms growth with azithromycin, the following hypothesis can be formulated.

First, we can postulate activation of adhesion factors biosynthesis as observed in *A. baumannii* under the action of subinhibitory concentrations of the antibiotic imipenem (Nucleo et al., 2009). Secondly, we can suggest that azithromycin should have a positive influence on the synthesis of quorum sensing regulatory factors necessary for the formation of biofilms, as shown for macrolides in the case of *Chromobacterium violaceum* (Liu et al., 2013). Finally, we cannot exclude an effect on the regulatory systems involved in cyclic diguanosine monophosphate (c-di-GMP) biosynthesis controlling biofilm matrix production, as demonstrated for aminoglycosides in the case of *E. coli* (Boehm et al., 2009). Thus, antibiotics not only cause death or microorganisms growth inhibition, but should act as stress response inducers at subinhibitory concentrations (Linares et al., 2006).

We demonstrated a correlation between the ability of pseudomonads to synthesize AHL and the activity of azithromycin on biofilm formation. It is shown that the effect of activating the growth of biofilms is accompanied by an increased synthesis of polysaccharide matrix components. Therefore, it can be assumed that the activation effect is most likely due to the combined action of several regulatory mechanisms (quorum sensing system, as well as probably c-di-GMP dependent systems) involved in the biofilm matrix formation process.

Of special interest is the inhibitory effect of ultralow concentrations of azithromycin on the formation of biofilms in studied pseudomonads. To explain it, we put forward the following hypothesis. The matrix of biofilms is a complex, multicomponent system of biopolymers (DNA, proteins and polysaccharides), but since azithromycin at extremely low concentrations does not affect the synthesis of DNA or proteins, it can be assumed that its main target should be the polysaccharides synthesis. Several polysaccharides participate to the construction of biofilms in pseudomonads. If the synthesis of one of them is suppressed by azithromycin in ultra-low concentrations, and the synthesis of the others is activated by azithromycin at higher concentrations, and both synthesis occur simultaneously, then the biofilm formation pattern presented in Figures 10 and 11 should be observed: inhibition should occur as the antibiotic suppresses synthesis of the first component, whereas the synthesis of the second one is not yet activated. At higher concentrations, azithromycin should activate the synthesis of the second component and compensate for the inhibition of the first one. However, this hypothesis is speculative and needs experimental confirmation.

These results demonstrate that the stimulation of the growth of biofilms by antibiotics is a universal phenomenon and is not only specific of human commensal microorganisms or pathogens, but is also shared by saprotrophs.

The next part of our work was the study of the effect of non-antibiotic drugs on the growth of biofilms. For that we decided to choose an anthelmintic preparation of niclosamide, for which

we first tested its ability to influence the growth of biofilms, and secondly the possibility of its use in combination with antibiotics to generate a binary antibiofilm preparation. In the composition of such a formulation, niclosamide should act by enhancing the effect of the antibiotic. Previous works have been realized on the inhibitory effect of niclosamide on the growth of microorganisms (Imperi et al., 2013; Torres et al., 2016), but the novelty of our work was that it was realized over a wide range of strains. In our study, the efficacy of niclosamide as an antibiofilm drug of a broad spectrum of action was demonstrated (data were not given in this work). The results allow to consider niclosamide as a promising component of complex antimicrobials (in combination with antibiotics). The interest of this strategy is also supported by economical reasons. Indeed, niclosamide is produced in large quantities over the world and in Russia in particular, and this makes possible the creation of complex antibiofilm medicines at a limited cost. Moreover, niclosamide has a very high activity: hundredths of a  $\mu\text{g/ml}$  reduce the number of metabolically active cells in biofilms by 50%. Finally, niclosamide is not toxic in those concentrations for the host organism.

We have also shown that niclosamide can potentially improve the action of antibiotics. The additive effect of the interaction of niclosamide and azithromycin may be associated with differences in the mechanisms of their antibiofilm action. It is known that azithromycin inhibits protein synthesis at the translational stage, interacting with the 50S subunit of the ribosome (Bakheit et al., 2014). The decrease of biofilm formation should be attributed to a deficiency of the necessary enzymes. In the case of niclosamide, as we have already noted, an inhibitory effect on the QS system of *P. aeruginosa* was described leading to a reduction of AHL synthesis (Imperi et al., 2013). However, niclosamide was also shown inhibiting biofilm formation in Gram-positive bacteria (Torres et al., 2016), which fundamentally differ of QS system. According to our experimental results, niclosamide affected the processes associated with the adhesion of planktonic cells to surfaces but the molecular mechanisms of action of niclosamide on the formation of biofilms require further researches.

The next stage of the work was to investigate the effect on the growth of cutaneous microorganisms biofilms of compounds which have no clinical applications but can also affect the development of the microbiota— such as cosmetic products and their components. Investigations of the influence of active cosmetic components on bacteria are currently abundant in international journals. This is due to the recent interest of cosmetic industry for the skin microbiota motivated by the emergence of cosmetics free or low level preservatives cosmetics under the influence of consumers. Indeed, preservatives are the causes of allergy and other skin disorders (Herman et al., 2013; Nabavi et al., 2015) but preservative free products are particularly sensible to contaminants and skin bacteria during storage and application.

Firstly, we studied the effects of PS291® and Uriage™ thermal water (UTW) on the growth of cultures, biofilms and surface properties of *S. aureus* MFP03 and *C. acnes* cells. Secondly, in confocal microscopy, we showed a change in the structure of *C. acnes* and *S. aureus* MFP03 biofilms under the influence of these substances. Thirdly, we hypothesized the most probable mechanism of the inhibitory effect of these compounds on the formation of *C. acnes* and *S. aureus* MFP03 biofilms at the stage of adhesion. Finally, proceeding from the fact that both thermal water and PS291® practically equally inhibit the growth of biofilms of representatives of two bacterial phylums, we can assume the existence of a universal mechanism of the inhibitory effect of these compounds on Gram-positive immobile commensal bacteria of human skin.

PS291® and UTW are without toxic effect on bacterial planktonic cells but significantly reduced the total biomass of their biofilms. These results were obtained both by CV staining and by CLSM observations. It is interesting to note that physiological water (PS) in the same concentrations not only did not have a significant effect on the growth of the biofilms, but, moreover, removed the suppressive effect of PS291®. This indicates that the cause of the inhibitory effect of thermal water on biofilms *C. acnes* and *S. aureus* MFP03 is determined by its chemical composition

It is also possible that in the case of PS, as well as with thermal water, at a high concentration (i.e., with a strong dilution of the medium), processes that increase the resistance of biofilms to the effects of unfavorable factors can be triggered, like in *E. coli* in the case of resistance to ofloxacin (Bernier et al., 2013). This may explain the removal of the PS291® effect in the presence of PS, as well as the absence of a pronounced additive effect from the combination of PS291® and thermal water. At the same time, thermal water at high concentrations (30% or more) did not stimulate the growth of biofilms. However, this hypothesis is speculative and needs further verification.

PS291® or teflosa (from "coating like Teflon", [http://www.solabia.com/Produto\\_16,1/Cosmetique/Teflose.html](http://www.solabia.com/Produto_16,1/Cosmetique/Teflose.html)), according to the manufacturer, is able to form a layer on surfaces that theoretically interferes with adhesion. The prevention of cell adhesion and the formation of biofilms by teflosa was previously observed using the non-acneic strain *C. acnes* BEC1666 (Enault et al., 2014). This is confirmed by the example of *S. aureus* MFP03 and two acneic strains of *C. acnes*, which indicates the absence of dependence of this effect on strain differences and the acneic phenotype. PS291®, firstly, significantly reduces the adhesion of *S. aureus* cells to the glass and the formation of microcolonies; secondly, it changes the properties of the surface of the cells of both microorganisms. *S. aureus* cells becomes more electronegative, and in both strains of *C. acnes* the affinity for polar organic solvents decreases. It is likely that

PS291® binds to surface structures of cells, which changes and reduces their ability to adhere. However, this assumption requires confirmation.

As for the biofilm architecture, in this case both thermal water and PS291® have a similar effect. In their presence, the biofilms of both microorganisms are thinned, and the number of outgrowths and structural formations on the surface decreases, i.e. in fact, the three-dimensional structure of biofilms is simplified. This confirms the hypothesis of the antiadhesive properties of tested substances: cells in biofilms in the presence of teflosa or UTW are more easily dispersed. In *S. aureus* MFP03 at 50% UTW in medium, the biofilm is actually a monolayer of cells that differs little in thickness from that after the stage of initial adhesion. In *C. acnes*, probably because of the higher hydrophobicity of cells, this effect is not so pronounced, but it is also present.

Studies of the effects of UTW and teflosa on *S. aureus* MFP03 and *C. acnes* are also important in connection with the recent discovery by molecular methods of the presence in the microbiota of sores and acne vulgaris of significant quantities of *Staphylococcus* spp. along with *C. acnes*, which suggests a possible involvement of staphylococci in the development of acne (Dreno et al., 2017). In model experiments of binary biofilms, both microorganisms are able to grow both under anaerobic and aerobic conditions (Tyner & Patel, 2016). Understanding the processes occurring on the skin during acne development, as well as during exposure to cosmetics, will allow to develop drugs or cosmetics that could reduce the impact of acne which affects a large number of people over the world. It should be noted that according to the regulations, particularly in UE, anti-acneic treatments can be considered either as cosmetics or pharmaceutical compounds.

To summarize, it can be said that the study of the effect of cosmetic products and their components confirmed that they can have a significant effect on the growth of biofilms of human commensal microorganisms. Without cytotoxic action, PS291® and UTW are able to modify the processes of cell adhesion to each other and to the surface. The absence of -toxicity for microorganisms at an effective concentration is an important characteristic of these compounds, as this means that it is possible to find ways to control human microbiota and its biofilm formation not only with drugs or substances directly affecting the metabolism of microorganisms, but also with compounds whose addition in a medium does not change the general metabolic state of microorganisms. Thus, such compounds should be safe for humans but in addition should not promote bacterial resistance by exerting a very limited evolutionary pressure.

Finally, the last group of active compounds studied in this work was NUPs. NUP are endogenous compounds that have a wide range of functions in the body. We demonstrated for

the first time that A and C types of NUP have a significant effect on skin microorganisms and their relationships in binary communities. It is especially important to note that the known functions of NUPs are not as antibacterial substances but, more likely, as intercellular communication signals, not only between host eukaryotic cells, but also between the host organism and its skin microbiota. As a fact, these peptides, without having generally a significant effect on the growth of planktonic cultures, affect the formation of both mono-species biofilms and the competitive properties of microorganisms in binary biofilms.

NUPs are synthesized by cardiocytes as hormone (ANP) and also by endothelial cells of the capillary vessels (CNP). Produced and transported by blood in different areas of the body or secreted locally in the tissues, such as in skin, they exert their functions in different conditions of in temperature and concentration of oxygen. Within the human body, the temperature is approximately 37° C and both aerobic and anaerobic zones exist. The skin is also presenting aerobic and anaerobic microniches, but the temperature in skin is significantly lower. In particular, on the skin of the forehead, where acne usually develops, it is about 33 ° C (Boutcher et al., 1995). Inside the hair follicle, peptides can directly diffuse from blood and surrounding tissues and affect the community of microorganisms, including *C. acnes* and *S. aureus* which are abundant in oily regions such as the hair follicle. This impact is very significant for two reasons. First, in a special zone of the follicle designated as “bulge”, blood capillaries form a dense network (Xiao et al., 2013) and the concentration of NUPs can be particularly high. Secondly, in this region, microorganisms are at a very small distance from the capillaries, thus actually undergo a constant effect of the peptides.

*C. acnes* is one of the dominant components of the skin microbioma and presumably involved in the development of acne. However, hundreds of microorganisms inhabit the skin glands and hair follicles, therefore it is excessive to consider that *C. acnes* is the only causative agent of acne. Recent studies show that in some cases, *S. aureus* is also abundantly present in the inflamed sores of acne (Totte et al., 2016, Dreno et al., 2017). So far, no direct evidence has been found for the role of *S. aureus* as a causative agent of acne, however, there are many reasons to assume its involvement in the inflammatory process. Hence, *S. aureus* abundantly colonizes skin areas affected by psoriasis and atopic dermatitis (Elfatoiki et al., 2016, Lacey et al., 2016), causing inflammation and complicating the treatment process. Within the cavities of the skin glands and hair follicles, *C. acnes*, being to some extent an aerotolerant anaerobic, prefers to occupy anaerobic micro-niches (Matard et al., 2013), which forms biofilms that can include *S. aureus*.

The biofilm-specific effect of NUPs can be explained by the fact that in the human body, microorganisms live predominantly in the form of multi-species biofilms. This especially applies



to the skin, where the physical and chemical parameters of the environment simply do not allow the development of planktonic culture. We have shown that NUPs have not only a large number of regulatory functions within the human body but are also capable of affecting human commensal microorganisms, which broadens the horizon of our understanding of the work of the human hormonal system. In addition, we are one step closer to understanding the global picture of the interaction of commensal microbiota with the human body.

The difference in the strength of the effect and its dependence on the peptide concentration in different microorganisms can be an evolutionary adaptation of the human hormonal system to interaction with the microbiota. An illogical, at a first glance, inverse dose dependent effect of NUPs on *S. aureus* can be explained, first, by the fact that at high concentrations NUPs are able to form aggregates, which can lead to a decrease in their activity (Torricelli et al., 2004) and secondly, as *S. aureus* lives in places where the concentration of the peptides should be normally lower than in the case of *C. acnes*. Then *S. aureus* should have adapted to detect low concentration of NUPs. This hypothesis is supported by the fact that both inhibitory and stimulatory effects in the case of *S. aureus* are stronger at a low concentration of NUPs. *C. acnes* is concentrated in areas where the concentration of peptides is presumably higher, and this bacterium appears more responsive at high NUPs concentrations. Nevertheless, NUPs appear to have a strain-specific effect on *C. acnes*: when the growth conditions are changing, the nature of the effects of NUPs on the different strains of *C. acnes* vary. This may be a consequence, on the one hand, of the closer evolutionary interaction of the endocrine human system with *C. acnes* and, perhaps, the more long and close coexistence of *C. acnes* with human, and on the other hand of the different roles of *C. acnes* strains on the human skin and their different interactions with the body.

Before investigating the effect of active compounds on binary biofilms, we investigated the interactions of microorganisms with each other in binary systems without the addition of active compounds - cosmetics or NUPs. We have shown that in binary biofilms of two staphylococci, *S. aureus* MFP03 has a significant competitive advantage over *S. epidermidis* MFP04. And if *S. aureus* MFP03 is able to suppress the growth of *S. epidermidis* MFP04 by approximately two orders of magnitude in the case of simultaneously formed biofilms and actively colonizes mature 24-hour biofilms of *S. epidermidis* MFP04, then *S. epidermidis* MFP04 is able to withstand the presence of *S. aureus* MFP03 only when pre-formed mono-species mature 24-hour biofilms. The colonization of mature biofilms of *S. aureus* MFP03 by this strain is much more difficult. At the time of this dissertation, only one paper by Vandecandelaere et al., 2017, focused on binary biofilms of a number of strains of *S. aureus* and *S. epidermidis*, was found. It shows that in a binary biofilm, both microorganisms change the expression level of a number of genes, and *S.*

*aureus* Mu50 strains generally showed less metabolic activity in the presence of *S. epidermidis* strains, while expression of resistance genes to a number of antibiotics in *S. epidermidis* strains increased. Moreover, the ratio of the number of CFU in binary biofilms remained the same as at the beginning of the experiments and did not change with the growth of the biofilms was observed. When comparing the results of our work with the work of Vandecandelaere et al., 2017, it is evident that the interaction of *S. aureus* and *S. epidermidis* is strain-specific. In the work of Vandecandelaere et al., the *S. epidermidis* strains was isolated from a tracheal implant biofilms, and the *S. aureus* strains were isolated either from skin wounds, or implant surface. Collection strains were also employed. This suggests that, perhaps, on the skin when coexisting in close or identical microniches of *S. aureus* MFP03 has an advantage over *S. epidermidis* MFP04, since both microorganisms were isolated from the skin of healthy volunteers.

Binary biofilms of *S. aureus* and *C. acnes* were previously only studied by Tyner & Patel, 2016. In this work, the authors showed that *S. aureus* IDRL-4284 survived better as a part of the binary biofilm with *C. acnes* IDRL-7676 than in mono-species biofilms with long-term (more than 10 days) cultivation. In general *C. acnes* had a beneficial effect on *S. aureus* (both strains were from clinical origin). At the same time, the number of CFU of *C. acnes* did not change in the presence of *S. aureus*. The data obtained in this work are coherent with the results of the work of Tyner & Patel, because at 37° C. *S. aureus* MFP03 in the presence of both strains of *C. acnes* grew better than in mono culture. At the same time, probably because of its growth rate, *S. aureus* MFP03 won the competition with *C. acnes* in the case of simultaneously formed biofilms. Our results correlate with the data of Wollenberg et al., who showed that coproporphyrin III, synthesized by *C. acnes*, stimulates the aggregation of *S. aureus* cells, its adhesion to the surface and the growth of biofilms (Wollenberg et al., 2014). However, in our experiments at 33 ° C the picture changed: in all the cases where *S. aureus* MFP03 had an advantage in growth rate or in the form of an already preformed biofilm (simultaneously formed biofilms or biofilms created on the basis of preformed mono-species biofilms of *S. aureus* MFP03 ), the presence of *C. acnes* RT5 did not significantly affect the number of CFUs of *S. aureus* MFP03. Whereas in the case of binary biofilms, a significant decrease in the number of CFU of *S. aureus* MFP03 was observed on the basis of preformed mono-species biofilms of *C. acnes* RT5, *C. acnes* biomass RT5 was not significantly changing. This indicates, first that there is a strain specificity in the interactions of microorganisms, and, secondly that there are possible ecological interactions of strains on the skin: *C. acnes* RT5, at the normal skin temperature (33° C), is apparently able to suppress the growth of *S. aureus* MFP03.

If we talk about the studies of binary biofilms of *S. epidermidis* and *C. acnes*, we find only one paper by Eroshenko et al., 2017, devoted to the effect of N-acetylcysteine on a number of

Gram-positive skin microorganisms, in which the authors tested the effect of the compound on mono-species biofilms, as well as on binary biofilm of *S. epidermidis* and *C. acnes* (Eroshenko et al., 2017). The authors suggested that since *S. epidermidis* is more resistant to the action of N-acetylcysteine than *C. acnes*, the long use of this drug can alter the balance of microorganisms on the skin (Eroshenko et al., 2017). There are some papers that report an antagonism of *S. epidermidis* and *C. acnes* (Christensen et al., 2016). This was detected by seeding on Petri dishes with subsequent analysis of the zones of enlightenment, genome sequencing and identification of certain genes responsible for the synthesis of antimicrobial compounds. Our work showed that the investigated strains of *S. epidermidis* MFP04 and *C. acnes* RT4 and RT5 behave differently in binary biofilms. *S. epidermidis* MFP04 at 37 ° C in the presence of the strain RT5 grew less than in mono-species biofilms, whereas in the presence of the strain RT4 it was the opposite and it grew better. This shows again the strain-specific interactions of these species. As in the case of *S. aureus* MFP03, both strains of *C. acnes* grew worse in the presence of *S. epidermidis* MFP04 than in mono-species biofilms, as *S. epidermidis* grew better under anaerobic conditions than *S. aureus* MFP03. This work shows that in some cases there may be an antagonism between the strains of *S. epidermidis* and *C. acnes*, similar to the one observed in Christensen et al.

We investigated the effect of UTW and PS291® on the growth of binary biofilms of *S. aureus* MFP03 and *C. acnes* RT5, as well as *S. aureus* MFP03 and *S. epidermidis* MFP04. The addition of UTW to the culture medium increased the competitive properties of *S. epidermidis* MFP04 in binary biofilms with *S. aureus* MFP03 compared to the control, and also increased the competitive properties of *C. acnes* RT5 in binary biofilms with *S. aureus* MFP03. PS291® had a different effect: in *S. aureus* MFP03 and *S. epidermidis* MFP04 binary biofilms, it reduced the growth of *S. epidermidis*, and in *C. acnes* RT5 + *S. aureus* MFP03 binary biofilms it increased the competitive properties of *C. acnes* RT5, partially inhibiting the growth of *S. aureus* MFP03. This can be one of the reasons for the beneficial effect of UTW and PS291® on the skin, as they disrupt the growth of biofilms of *S. aureus* considered to be a more aggressive pathogen. It can be noted that in the case of *C. acnes* RT5 and *S. aureus* MFP03 binary biofilms, the effect of both cosmetic products differs from that in mono-species biofilms, where both compounds suppress the growth of biofilms of both microorganisms.

In the case of the effect of NUPs on *C. acnes* RT5 and *S. aureus* MFP03 binary biofilms, as in the case of UTW and PS291®, the effects of ANP and CNP differ from those in the case of single-species films. The close proximity and interaction of the two microorganisms critically changes the nature of the effect of the peptides on them. Both peptides begin to stimulate the growth of *C. acnes* biomass whereas there has been significant growth inhibition in mono-species biofilms. This may indicate the existence of mechanisms of interspecies interaction and,

possibly, mutualism that protect *C. acnes* from the action of the peptides in the presence of *S. aureus*, or allow it to reverse their presence in its favor. Based on the data obtained by us during this work, we can formulate a hypothesis on how NUPs regulate the composition of the microbial community in biofilms. Under normal conditions (healthy skin, temperature 33° C) within the community where there are *C. acnes* and *S. aureus* NUPs enhance the competitive advantage of *C. acnes*, suppressing the growth of *S. aureus*. This seems logical, because *S. aureus* is a more aggressive species and a pathogenic microorganism whose excessive growth is highly undesirable (Vandecandelaere et al., 2017). Under certain conditions (clogging of pores, formation of comedones), the environment becomes more favorable for an overgrowth of *C. acnes*. There is a focus of acne and an inflammatory process begins, which is accompanied by a local increase in temperature (rises to a level of 37° C). As the temperature rises, the situation changes - *S. aureus* and *C. acnes* seem to shift to mutualistic interactions, and their coexistence in biofilms has a beneficial effect on growth: both microorganisms begin to multiply intensively, which exacerbates the situation in the inflammatory focus. And it is at the stage of increased local temperature that NUPs begin to inhibit the growth of biofilms of both microorganisms, which allows the human defense systems to cope with inflammation.

We can also suggest the hypothesis of an involvement of A and C type NUPs in the development of psoriasis. According to the research, during the development of psoriasis on affected skin areas conditions are favorable for an abundant growth of *S. aureus*. Since psoriasis presumably has an autoimmune nature and usually proceeds in a wavy manner, we can presume the participation of NUPs in the course of its development. At the stage when the psoriatic plaques are not yet inflamed and the temperature is normal for the skin, *S. aureus* incomes and begin to proliferate intensively. Since, for reasons that are not yet clear, in the field of psoriatic plaques, *S. aureus* appears to have greater competitive advantages over other microorganisms and it can be assumed that in some microniches it forms practically mono-species biofilms. On these biofilms, NUPs have a stimulatory effect on the pre-inflammatory stage, which may be one of the causes of inflammation. In the process of inflammation, the local temperature also rises, which leads to the launch of the reverse process: NUPs begin to inhibit the growth of *S. aureus* biofilms. This hypothesis agrees with experimental data: the inhibition of growth of *S. aureus* biofilms at 37 ° C with natriuretic peptides occurs at relatively low concentrations of the peptides in the medium. At the same time, it is known that excessive psoriasis of the skin leads to excessive angiogenesis. More capillaries mean greater blood circulation and a greater concentration of available NUPs. With the increase in the number of capillaries on the inflamed skin area, the concentration of the peptides should increase i the tissue and then counteractthe inhibitory effect. Of courser, in order to finally confirm both hypotheses, *in vivo* studies should

be necessary.

If we consider the binary systems of *S. aureus* MFP03 and *S. epidermidis* MFP04, then it seems to us very significant that at 37° C ANP and CNP stimulate the growth of mono-species biofilms of *S. epidermidis*, whereas in the case of mono-species biofilms of *S. aureus* on the contrary at 37 ° C both peptides, predominantly suppress their growth. These facts allow to refine the hypothesis about the regulatory and, possibly, protective role of NUPs in human skin. Under normal conditions (when there is no inflammation and the temperature is about 30-33 ° C, depending on the zone on the skin), the growth of *S. epidermidis* (biofilms and plankton cells) is slightly suppressed or does not change under the influence of NUPs. If the balance of the microorganisms on the skin is disturbed, the much more dangerous and aggressive *S. aureus* begins to multiply intensively, which leads to inflammation and, accordingly, to a local (or general) rise in temperature to 37° C and higher. In this case NUPs begin to suppress the growth of *S. aureus* and stimulate the growth of not only of *C. acnes*, but also of *S. epidermidis*. Thereby, the risk of complications and additional deterioration of the condition caused by an excessive reproduction of *S. aureus* should be reduced, and the normal ratio of microorganisms to the skin may be restored. Thus, it is possible to explain the protective role of *S. epidermidis* for the human skin, which is becoming increasingly known (Iwase et al., 2010, Sugimoto et al., 2013) if it is taken in consideration that *S. epidermidis* receives additional help from the human endocrine system by counteracting the aggressive strains of *S. aureus*. This hypothesis is speculative, nevertheless, the results of our research allow to consider with sufficient justification that NUPs are important components of the system of interaction of the microbiota with the skin and the human body. This is opening new perspectives both in fundamental aspects of dermatology, microbiology, and physiology, and in their applied aspects: cosmetology and pharmaceuticals. They also testify to the profound evolutionary relationship between human and his microorganisms-commensals.

An important part of this work was the study of the biochemical composition of the matrix of *C. acnes* biofilms. At the time of this dissertation, two papers on the composition of the *C. acnes* matrix were published. In the first one (Jahns et al., 2016), the matrix was studied using specific fluorescent dyes for DNA, polysaccharides, and proteins. Using fluorescent microscopy, the authors determined the presence of carbohydrates, proteins and DNA in the matrix without further deepening into the biochemical structure of the compounds. Another work by Okuda et al., 2018 was devoted, among other things, to the isolation of the matrix and the analysis of its composition. The authors estimated the amount of DNA, proteins and poly-N-acetylglucosamine (surface adhesin). Their data are close to those obtained in the present work. The authors also characterized some matrix proteins, for example, hydrolases, enolase, lipoprotein RlpA and

hemolysis factor CAMP. However, the method by which they received biofilms is doubtful, since Chiba et al., 2015, by whose method they isolated the matrix, cultivated the culture in a liquid medium in test tubes under static conditions without a specialized carrier, after which all the biomass was precipitated, washed and the matrix isolated. It is not entirely clear whether pure biofilms or biofilms with planktonic were actually obtained in this case, because, in our experience with *C. acnes*, these microorganisms form biofilms on the bottom of plastic tubes very poorly.

In our work, a deeper and more comprehensive study of the *C. acnes* RT5 matrix was carried out. Firstly, we obtained mature (according to Tyner & Patel, 2016) 7-day biofilms on the surface of filters on a dense medium, which eliminated the material of the nutrient medium and made it possible to isolate the matrix of biofilms. Secondly, we did not treat the biofilm with dispersin B and DNase, which automatically allowed us to analyze the polysaccharide composition of the matrix. Third, *C. acnes* extracellular hydrolases were known long before Okuda et al., 2018 (Holland et al., 2010). Our technique allows us to isolate the matrix of biofilms without changing the chemical structure. Also, for the first time we calculated the quantitative ratio of the main components of the matrix.

In the matrix of *C. acnes* RT5, isolated by our method, most of the organic carbon (54%) belongs to the carbohydrate component. The second component is peptides: 11.9%. DNA accounted for about 2.8% of the total organic matter of the matrix. The remaining organic matter, presumably contains intermediates metabolites and first of all, intermediates in porphyrins synthesis. Proteomic analysis of the biofilms matrix showed that it contains more than 400 proteins. Enzymes are very diverse, indicating a high catalytic potential of *C. acnes* matrix. Many of the proteins are proteins of the cytosol and probably enter the matrix following autolysis of a part of the population as, normally occurring during the growth of biofilms. However, there is a high probability that these intracellular proteins within the matrix have certain unknown functions that are different from those inside the cell and are not a "dead weight". For example, dozens of hydrolases, which are specific for different substrates, probably allow *C. acnes* biofilms to dissolve polymers of surrounding tissues in the host organism, in particular, human skin, which causes their pathogenicity and probably determines the role of *C. acnes* in the development of acne and other diseases. Chaperones and chaperonins can participate in the organization of the matrix carcass, together with ribosome particles and whole ribosomes. We do not know in what form the ribosomes are represented in the matrix: in the form of whole particles, in the form of separate subunits, protein aggregates or proteins separately. Moreover, out of more than 50 ribosomal proteins we detected only 43 of them. We can assume that the ribosomes are represented in the matrix in all states, however, their potential

role and the role of their proteins in the matrix of biofilms is still unclear.

We conducted the SERS analysis of the matrix of *C. acnes* biofilms for the first time. Analysis of the SERS peaks also confirms the isolation of the biofilm matrix, since more than 40 peaks in the matrix and biomass of the cells were distinguished. The most intense peaks of both phases of the matrix are at 370-386  $\text{cm}^{-1}$ . However, the spectra of SERS alone does not carry information about the nature of matrix substances, since there is currently no SERS database of organic compounds. Therefore, SERS is advantageously used in combination with other techniques, such as mass spectrometry and NMR. Nevertheless, we can state that we first made typing of biofilms and *C. acnes* matrix by the SERS method, which will help in the future, for example, to determine the presence of *C. acnes* signatures in complex biological samples by the comparatively fast and easy SERS method.

### Conclusions

1. For the first time it has been shown that the antibiotic azithromycin in subinhibitory concentrations stimulates the growth of biofilms of the saprotrophic strain of *P. chlororaphis* 449, and that the AHL-dependent quorum-sensing system participates in this process. This fact gives the possibility of suppressing of QS-system functioning to prevent the stimulating effect of antibiotic, which is dangerous in case of pathogenic bacteria. It is established that the stimulation of the growth of biofilms by azithromycin, first of all, is expressed in the increased synthesis of matrix polysaccharides, which leads to increased resistance of biofilms preformed in the presence of azithromycin to heat shock.

2. The inhibitory effect of the anthelmintic agent niclosamide on the growth of plankton cultures and biofilms of *S. aureus*, *M. luteus* C01 and *K. schroeteri* H01 was first discovered. Precisely, niclosamide antagonize the stimulatory effect of subinhibitory concentrations of azithromycin on the growth of *S. aureus* 209P biofilms. This makes it promising for use in complex preparations with this antibiotic.

3. It is shown that the UTW <sup>TM</sup> and PS291® used in cosmetics significantly suppress the growth of *S. aureus* MFP03 and *C. acnes* biofilms, without showing a toxic effect. UTW displaces the balance of viable bacteria towards *S. epidermidis* MFP04 in binary biofilms of *S. aureus* MFP03 and *S. epidermidis* MFP04. In binary biofilms of *S. aureus* MFP03 and *C. acnes* RT5, both compounds shift the balance towards *C. acnes* RT5, which is favorable for the condition of the skin, as *S. aureus* is a dangerous pathogen.

4. The dependence on the conditions of cultivation of the human NUPs effect on the growth of biofilms of *S. aureus*, *S. epidermidis* and *C. acnes* RT5 was first established, which

can correlate with different skin conditions. It has been shown that NUPs regulate the composition of binary biofilms of skin commensals: enhance the competitive advantages of *S. epidermidis* and *C. acnes* RT5 over *S. aureus*. These results allow to suggest the use of NUPs or NUPs analogs in cosmetology and dermatology to normalize the balance of the skin microbiota.

5. A technique for isolating the matrix of the biofilms of Gram-positive bacteria has been developed. The ratio of the biochemical components of the matrix of *C. acnes* RT5 biofilms was studied for the first time. It was shown that the dominant component of the matrix of biofilms of *C. acnes* RT5 are polysaccharides.

6. For the first time, an in-depth study of the total proteome of the *C. acnes* RT5 matrix was carried out. The presence of more than 400 proteins in the matrix, the presence of a large number of hydrolase enzymes specific for various substrates, and other enzymes provides a high catalytic potential to the matrix of *C. acnes* RT5 biofilms.

7. SERS analysis of biomass and biofilm matrix of *C. acnes* RT5 was carried out for the first time, the spectral profile of cells and of the matrix of *C. acnes* RT5 was compiled, which will allow using it in future to create a database of SERS spectra of biological samples.



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