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Study on the effect of blackcurrant extract – based preservative on model membranes and pathogenic bacteria

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ABSTRACT

In this work the cosmetic preservative based on a Ribes Nigrum (blackcurrant) plant extract (PhytoCide Black Currant Powder *abbr*. BCE) was investigated to evaluate its antibacterial effect and to gain an insight into its mechanism of action. The influence of this commercially available formulation on model *Escherichia coli* and *Staphylococcus aureus* lipid membranes was studied to analyze its interactions with membrane lipids at a molecular level. The mixed lipid monolayers and one component bacteria lipid films were used to investigate the effect of BCE on condensation and morphology of model systems and to study the ability of BCE components to penetrate into the lipid environment. The *in vitro* tests were also done on different bacteria species (*E. coli*, *Enterococcus faecalis*, *S. aureus*, *Salmonella enterica*, *Pseudomonas aeruginosa*) to compare antimicrobial potency of the studied formulation.

As evidenced the *in vitro* studies BCE formulation exerts very similar antibacterial activity against *E. coli* and *S. aureus*. Moreover, based on the collected data it is impossible to indicate which bacteria: Gram-positive or Gram-negative are more susceptible to this formulation. Model membrane experiments evidenced that the studied preservative affects organization of both *E. coli* and *S. aureus* model system by decreasing their condensation and altering their morphology. BCE components are able to penetrate into the lipid systems. However, all these effects depend on the lipid composition and monolayer organization. The collected results were analyzed from the point of view of the mechanism of action of blackcurrant extract and the factors, which may determine the activity of this formulation.

1. Introduction

Currently, there is a trend among consumers to look for cosmetics that are mainly composed of natural substances, which are considered to be safer for their health than synthetic compounds as well as being environmentally friendly. This attitude results from the awareness of the consequences of the climate change, the importance of the concept of sustainable development and the resulting desire to live more "greener" and healthier for themselves and for nature [1,2].

The fact is that many ingredients of cosmetics are known to be harmful to the environment and to living organisms. Some of them are not effectively removed in sewage treatment plants. They are characterised by high persistence, the possibility of bioaccumulation, and after reaching the aquatic and terrestrial environment, they pose a real threat to plants and animals. Moreover, they can also easily enter the food chain, which makes them harmful also to humans. Another problem is that some of these substances undergo chemical modifications (total or partial) under the influence of the sunlight, water or in the presence of other compounds. As a result, all living organisms are exposed to a wide range of chemicals whose toxic effects (e.g. hormone disruption, problems with reproduction, growth inhibition, neurotoxicity) are not always fully explored [3–6]. Taking into account the intensity of the use of cosmetics and personal care products, it is clear that huge amounts of more or less harmful substances are released into the environment every day. All this causes an increase in public interest in "green" cosmetics and focuses scientific research on the search for their "green" ingredients.

Many extracts and the essential oils derived from plants meet the

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criteria for eco-ingredients and they are frequently used in cosmetics as the active care components (e.g. moisturising or nourishing) or as necessary additives (e.g. preservatives). For example, *Camellia sinensis* extract acts as an anti-ageing substance [7], *Baccharis antioquensis* leaf extract has photoprotective potential and can be used as an active component of the natural sunscreens [8], while the Orchid flower is a safe and efficient ingredient in anti-ageing and whitening formulations [9]. More examples of the potential of various plant extracts in cosmetic industry were described in the literature [e.g. 10,11].

The experiments presented in this paper are a part of a broad group of studies aimed at searching for natural ingredients of cosmetics and exploring their mechanism of action and toxicity. PhytoCide Black Currant Powder (BCE) investigated in this work, is a commercially available formulation recommended for the use in cosmetics as an antimicrobial agent, which also has anti-inflammatory, soothing and moisturising properties. This product is Ribes Nigrum (blackcurrant) plant extract of a confirmed antibacterial and antifungal activity. Therefore, it can protect cosmetics and personal care products from microbial growth by acting as a natural preservative [12,13]. However, blackcurrant plant extract itself is known for a wide range of beneficial effects on human health, e.g. antioxidant, anti-inflammatory and antiseptic properties. It can also reduce the risk of certain non-communicable chronic diseases and it may be helpful in the case of vascular diseases since can regulate the blood lipid profile [14,15]. The foregoing properties of the extract are determined by its composition, which is highly complex and very variable (please see Experimental section).

There seems to be a great need for novel natural substances to protect products from microbial growth. Currently used synthetic preservatives (e.g., organic acids, alcohols, quaternary ammonium compounds) are controversial since they may cause various undesirable effects for consumers (toxicity, irritation, or sensitization) [6,16]. Extremely debatable is the application of parabens because so far, their effects on human health are not fully clear. On the one hand, there have been animal studies and *in vitro* tests on their toxicity, genotoxicity and the influence on the endocrine system, while on the other hand, this activity towards the human body has not been documented. Therefore, selected parabens are currently considered to be safe cosmetic ingredients, however, only when they are applied in the recommended doses [17].

Many plant extracts and essential oils have natural antimicrobial properties, such as pequi leaf extract (Caryocar brasiliense Camb), *Melaleuca alternifolia* oil or thyme essential oil. Unfortunately, their activity is often too low to provide sufficient antimicrobial protection for the cosmetic. On the other hand, they can be used in combination with other compounds in a given formulation making it safer and "greener" [10,11,18]. However, the practical application of any substance in a cosmetic preparation requires detailed investigations of the effects of its use. From the point of view of antimicrobial activity and toxicity, it is crucial to know the mechanism of action. The site of activity of many antimicrobial agents is the cell membrane of the pathogen. Therefore, in this work, the results of experiments on the effect of BCE on lipid monolayers that are models of bacterial membranes (*Staphylococcus aureus* and *Escherichia coli*) are presented. Our studies included also the monolayers formed from individual lipids used to prepare the models mentioned above and *in vitro* tests on selected bacterial strains. All the results obtained allowed us to analyze the effect of the studied extract on membranes and bacterial cells and to discuss them in the context of the mechanism of action of this formulation.

2. Experimental

2.1. Materials

In monolayer experiments synthetic lipids of high purity (≥99%) purchased from Avanti Polar Lipids Inc., USA were used, namely: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (POPG), 1′,3′-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (sodium salt) (TOCL), 1′,3′-bis[1,2-distearoyl-sn-glycero-3-phospho]-glycerol (sodium salt) (TSCL) and 1,2-dipentadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (sodium salt) (PG di-15:0). The stock solutions of the lipids were prepared in the mixture of chloroform/methanol (9:1 v/v) (HPLC grade, ≥99.9%, Aldrich). PhytoCide Black Currant Powder (BCE) was purchased from online cosmetic shop escent.pl [19]. According to the producer information this formulation is a 100% Ribes nigrum (blackcurrant) plant extract, rich in polyphenols, however, the formulation contains also citric acid (1.5–4%); it is suggested to be used in cosmetics in the level of 1.0–3.0% and it is claimed to be stable at pH between 3 and 8 [13]. In the literature detailed information on the composition of blackcurrant plant extracts from various origins can be found [14,20–22]. For example the berries extracts contain a high level of polyphenols (mainly anthocyanins and proanthocyanidis), which may constitute over a half of total dry mass of extract, however, it may contain also vitamin C, organic acids (e.g. citric acid), micro- and macronutrients and essential oils. However, the exact composition of extract depends on many factors and it is different for fruits and leaves, it depends on the applied extraction procedure as well as on the region of the plant growth, environmental stress, age of the plant [14,20–22]. The latter was analyzed in the Discussion section in relation to antimicrobial effect of extract. In any case, the preparation studied here is a complex mixture of compounds from the flavonoid group, known for their health-promoting properties, including antimicrobial activity.

BCE solutions were prepared by dissolution of the powder in phosphate buffer saline (PBS) (pH 7.4). The sodium chloride, potassium chloride, disodium hydrogen phosphate and sodium dihydrogen phosphate (the salts for preparation of PBS) were high purity compounds (>99%) purchased from Avantor Performance Materials Poland S.A. The concentration of salts in PBS was the following: NaCl 13.7 mM, KCl 0.27 mM, Na_2HPO_3 0.43 mM and NaH_2PO_3 0.15 mM. The ionic strength of the buffer was 15.0 mM. To prepare PBS and in all the experiments ultrapure Milli-Q water of conductivity 0.055 μS/cm was used.

2.2. Model membranes

As model bacteria membranes the mixed lipid Langmuir monolayers were used. These monolayers were composed of major lipid classes characteristic for *E. coli* (POPE, POPG and TOCL (75%, 20%, 5%, respectively, by mole %)) and *S. aureus* (PG di-15:0 and TSCL (58% and 42%, respectively, by mole %)) bacteria, respectively [23]. However, the experiments were performed also for one component lipid monolayers formed from POPE, POPG, TOCL, PG di-15:0 and TSCL.

2.3. Methods

2.3.1. Monolayer experiments

The surface pressure (π) – area (A) isotherms for one-component lipid films and for the mixed monolayers mimicking bacterial membranes were recorded both on PBS and on BCE solutions. The concentrations of BCE solutions were of: 1 μg/mL, 5 μg/mL, 10 μg/mL, 25 μg/mL and 50 μg/mL. The measurements were performed on KSV-NIMA Langmuir trough (total area = 275 cm^2) having two Delrin barriers enabling symmetrical compression of the monolayers. The trough was placed on an anti-vibration table. The monolayers were formed by spreading an appropriate volume of lipid solutions by using Hamilton microsyringe (±1.0 μL) on the subphase. The monolayers were left for 5 min and then they were compressed with the barriers speed of 10 cm^2/min. The surface pressure was measured with the accuracy of ±0.1 mN/m with the Wilhelmy plate made of filter paper (ashless Whatman Chr1) connected to an electrobalance. The experiments were performed at a constant temperature (20 °C), which was controlled thermostatically (±0.1 °C) by a circulating water system. All the measurements were repeated at least twice to obtain consistent results (the error for the area

per molecule does not exceed 0.2 A^2/molecule).

2.3.2. Penetration experiments

The penetration studies were done to verify the ability of the components of BCE to incorporate into the lipid monolayers. The films were spread on buffer subphase and compressed to desirable surface pressure. After equilibration to the initial surface pressure π_{in} BCE solution was injected into the subphase and the measurements of the surface pressure in time were started. During experiments, the subphase was continuously stirred. For the mixed monolayers the measurements for $\pi_{in} = 10$ and 30 mN/m and preservative concentration of 5 μg/mL; 10 μg/mL and 50 μg/mL were performed. For selected concentrations (indicated in the text) also the measurements at 20 mN/m were done. For one-component lipid monolayer the penetration studies for $\pi_{in} = 10$ and 30 mN/m at the highest investigated BCE concentration were done.

2.3.3. Brewster angle microscopy studies

The alterations in the morphology of the films caused by BCE were recorded in Brewster Angle Microscopy (BAM) experiments. Measurements were performed on a buffer subphase and on the highest BCE concentration (50 μg/mL). In these experiments, an UltraBAM instrument (Accurion GmbH, Goettingen, Germany) equipped with a 50-mW laser emitting p-polarized light at a wavelength of 658 nm, a 10 × magnification objective, polarizer, analyzer and a CCD camera was used. The spatial resolution of the microscope was 2 μm. The Langmuir trough and Brewster Angel Microscope were placed on a table (Standa Ltd., Vilnius, Lithuania) equipped with an active vibration isolation system (antivibration system VarioBasic 40, Halcyonics, Göttingen, Germany).

2.3.4. Antibacterial activity testing

Antibacterial activity of BCE was studied against five pathogenic bacteria: Gram-positive *S. aureus* and *Enterococcus faecalis*, and Gram-negative *E. coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa*. The strains were purchased from the American Type Culture Collection (ATCC), deep frozen and defrost before testings.

The antibacterial properties of BCE *in vitro* were evaluated in accordance to Hancock Lab microdilution procedure (Modified MIC Method for Cationic Antimicrobial Peptides. Available online: http://cmdr.ubc.ca/bobh/method/modified-mic-method-for-cationic-antimicrobial-peptides) with some modifications. The studies were performed in flat-bottom, polystyrene, 96-well microdilution plates (Nest, China). Initially, bacteria were cultured on Tryptic Soy Agar (Becton-Dickinson) for 24 h. Microbial inocula for antibacterial activity testing were prepared by suspending a few colonies in sterile distilled water. The densities of the inocula were adjusted to 0.5 McF with a densitometer (Biosan, Poland). Bacterial suspensions were subsequently diluted 1:200 in Miller Hinton Broth II (MHB) (Merk, Germany) and used for testing immediately after preparation. BCE powder was dissolved in water to obtain stock solution of 100 mg/mL from which series dilutions in water were made giving intermediate concentrations ranging from 0.01 mg/mL to 10 mg/mL. Microdilution plates were inoculated with 20 μL of the individual BCE solutions and 180 μL of bacterial suspensions in MHB. The growth control and sterility control wells contained water instead of BCE and sterile MBH instead of bacterial suspensions, respectively. Taking into account the volume of added bacterial solution and the volume of BCE solution the final BCE concentrations on the microplates were: 0.001 mg/mL, 0.005 mg/mL, 0.01 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 1 mg/mL, 10 mg/mL, and final bacterial inocula were approx. 1-2.5 × 10^5 CFU/mL. The plates were incubated without agitation at 35 ± 2 °C in ambient air for 24 h. The tests were performed in triplicate.

The results were read visually and with a microdilution plate reader (Tecan, Sunrise) by the measurement of the absorbance at 530 nm wavelength. The evaluation of antimicrobial properties was based on the percentage comparison of the growth of microorganisms in the presence of the tested compounds to the control growth without preservative.

The BCE minimal inhibitory concentrations (MIC) were defined as the lowest concentration that resulted in significant inhibition of bacterial growth compared to the growth control. After MIC readings, the contents of the wells were transferred on Tryptic Soy Agar (Becton-Dickinson) to determine bactericidal properties and the minimal bactericidal concentration (MBC) values which were the lowest BCE concentrations that caused complete inhibition of bacterial growth without any visible colonies on the agar surface after 24 h of incubation at 35 ± 2 °C in ambient air.

2.3.5. Monolayer data analysis

As the results of the compression of the monolayers the π/A isotherms were recorded. The shape and position of these curves were analyzed and the percentage shift of the isotherms (ΔA) caused by BCE was calculated and presented as ΔA *vs* BCE concentration plots. Based on the isotherms the compressional modulus (C_s^{-1}) values were also calculated [24].

$$C_s^{-1} = -A\left(\frac{d\pi}{dA}\right) \quad (1)$$

where *A* is the mean area per molecule at the given surface pressure.

The results of these calculations were presented as C_S^{-1} vs π plots. The values of the compressional modulus enable to define the state of the monolayer as follows: $C_S^{-1} < 2.5$ mN/m – gaseous (G); $C_S^{-1} = 12.5$–50 mN/m – liquid-expanded (LE); $C_S^{-1} = 100$–250 mN/m – liquid-condensed (LC); $C_S^{-1} > 250$ mN/m – solid (S) [24].

The penetration results were shown in the form of Δπ vs time plots, where Δπ is the differences between the experimental surface pressure and the initial surface pressure (π_{in}). When the $\Delta\pi > 0$ that is the surface pressure increases in time, the injected molecules penetrate the monolayer. When $\Delta\pi = 0$ no penetration occurs. Decrease of $\Delta\pi$ indicates desorption however, when the surface pressure drops below zero (that is below the initial surface pressure) it means dragging of the monolayer material to the subphase, leading to destabilization of the film [25].

From the experiments performed at different initial surface pressure the Δπ vs π_{in} curves were plotted. By extrapolating the regression of the plot to the x axis the maximum insertion pressure (MIP) was estimated. This parameter means the highest surface pressure, at which the insertion into the film occurs. Moreover, by adding 1 to the slope of the curves the synergy parameter can be calculated. The positive values of this parameter means a favorable binding of the substance to the monolayer [26].

2.3.6. Chromatographic analysis with mass spectrometric detection by LC-DAD-ESI-MS/MS system

To obtain more information on the composition of the studied BCE formulation, chromatographic and mass spectrometric analyses were performed.

For these analyses, an LCMS-8030 mass spectrometric system (Shimadzu, Kyoto, Japan) was used which consisted of LC-20ADXR HPLC pumps, an injector model SIL-20ACXR, PDA detector (photodiode array) model SPD-M20A, and mass selective detector, controlled with LabSolutions software version 5.60 SP1 (Shimadzu). The samples were eluted through a 150 × 4.6 mm i.d., 5.0 μm, Kinetex C18 chromatographic column preceded by a guard column of the same material (Phenomenex, Torrance, CA, USA). The injection volume was 10 μL, and the flow rate was 0.6 mL/min. The column was thermostated at 35 °C. A binary gradient elution was applied for the separation of the analytes. The mobile phases were: A - 1% formic acid in water and B - acetonitrile. The gradient profile was: (t (min), % B), (0, 9), (13, 35), (16, 90), (20, 90)). The mass spectra were recorded using the LC-MS system with electrospray ionization in the positive and negative ion modes. The ionization electrospray source with an electrospray voltage of 4.5 kV and a capillary temperature of 250 °C. N_2 gas as used for the spray. The system recorded total ion chromatograms, mass spectra, and ion

chromatograms in the selected ion monitoring mode (SIM) controlled by the LabSolutions software.

3. Results

3.1. The effect of BCE on the mixed films imitating bacteria membranes

The surface pressure-area (π/A) isotherms and the compressional modulus (C_S^{-1}) values in a function of the surface pressure (π) for model membranes of *E. coli* and *S. aureus* on a buffer and on BCE solutions are presented in Fig. S1 (Supplementary Materials). The flavonoid profile of the BCE extract obtained by chromatographic analysis with mass spectrometric detection in a LC-DAD-ESI-MS/MS system is presented in Table S1.

BAM images recorded for the model systems on a buffer and on BCE solutions are presented in Fig. 1. The percentage shift of the isotherms at 10 and 30 mN/m and a decrease of the compressional modulus values at 30 mN/m (in percent) are shown in Fig. 2.

The isotherm for *E. coli* model membrane registered during the compression of the film on a buffer subphase has a characteristic course (Fig. S1). The surface pressure starts to rise at ca. 105 Å^2/molecule and it increases monotonically up to ca. 43 mN/m where the characteristic bend in the curve is observed. At this region in BAM images (Fig. 1) the domain formation is detected. While the surface pressure is increasing the domains are growing till the film collapses at ca. 50 mN/m. Below the surface pressure of the domains formation the BAM images for *E. coli* monolayer are uniform. The maximum value of the compressional modulus for *E. coli* film on a buffer is ca. 120 mN/m, which means that the monolayer is in the Liquid Condensed state (LC) [23]. On the C_S^{-1} *vs* π curve the minimum at ca. 40 mN/m corresponding to the bend on the isotherm can be detected.

On the other hand, during the compression of *S. aureus* model membrane the surface pressure starts to increase at ca. 110 Å^2/molecule. When π reaches the value of 12 mN/m in the curve a plateau occurs. Then, the surface pressure increases monotonically up to ca. 60 mN/m where the film collapses. In the surface pressure range from ca. 0.5 mN/m to ca. 25 mN/m in BAM images the domains can be detected. These domains increase slightly in their number and size and above 25 mN/m the film becomes practically uniform. The plateau in the isotherm, reflected as the minimum in C_S^{-1} vs π plot, corresponds to the Liquid expanded (LE) - Liquid Condensed (LC) phase transition.

The results evidenced that BCE affects both *E. coli* and *S. aureus* model membranes. Namely, in the presence of the preservative the isotherms shift to the larger areas (Fig. S1, Fig. 2) and the morphology of the films is modified (Fig. 1). That means fluidization of the films caused by the molecules added into the subphase.

In the case of *E. coli* systems, at higher concentrations of BCE it is difficult to observe the changes in the curve position (the isotherms practically cover each other). Thus, the increasing concentration of the preservative does not strengthen its effect on the position of the isotherms. This is well noticed in Fig. 2. Namely, the percentage shift of the isotherm at 30 mN/m presented in the function of BCE concentration in the range of 5–50 μg/mL is practically constant. The results obtained at lower π (10 mN/m) evidenced nonlinear shift of the curve, and its stabilization in the range 25–50 μg/mL.

The BCE presence slightly affects the compressional modulus values for *E. coli* system (Fig. S1, Fig. 2). Namely, for 1 and 5 μg/mL the C_S^{-1} vs π curves are comparable to the one obtained for the monolayer on a

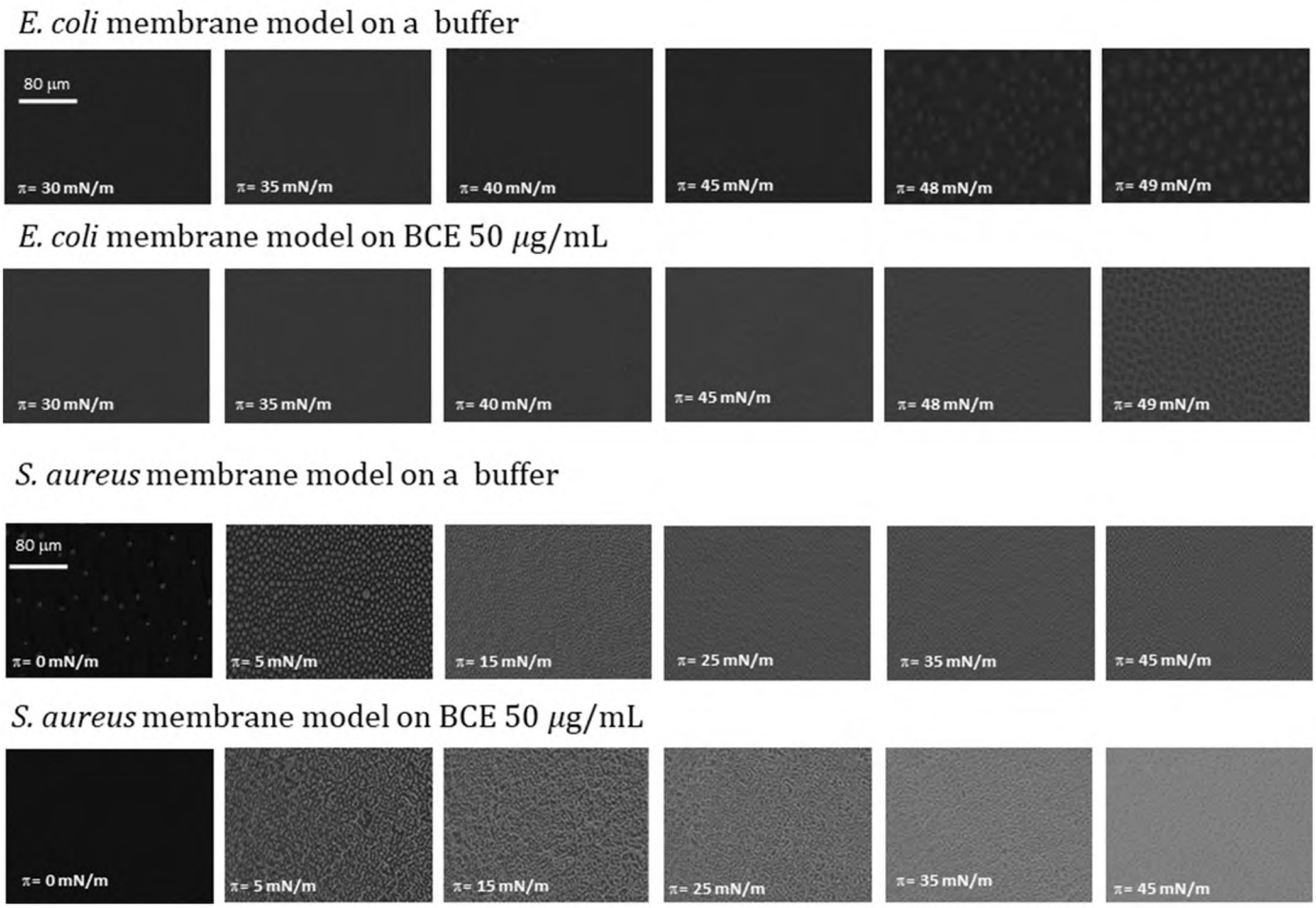

Fig. 1. BAM images for *E. coli* and *S. aureus* model membranes on a buffer and on BCE solution.

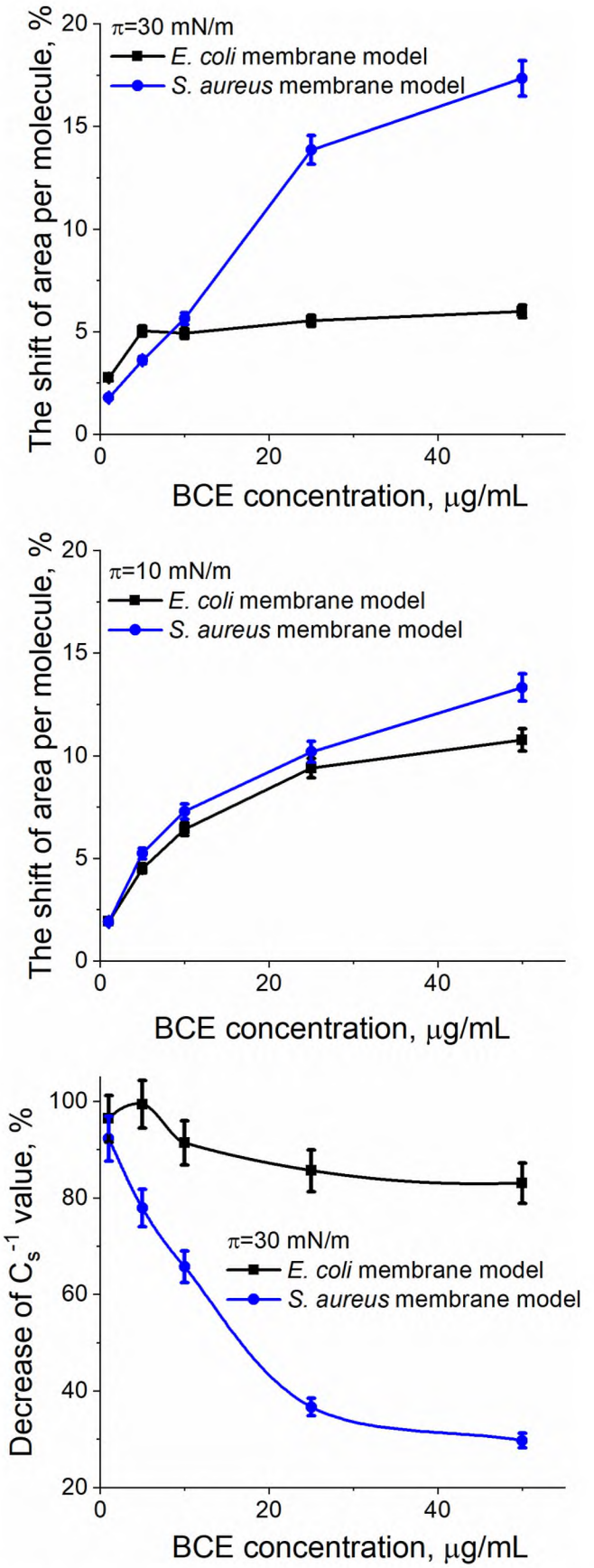

Fig. 2. The percentage shift of the isotherms for the films imitating model membranes in the presence of preservative calculated at 10 and 30 mN/m, and a decrease of the compressional modulus values at 30 mN/m.

buffer. Only at higher BCE concentrations (10, 25 and 50 μg/mL) the maximum values of C_S^{-1} are slightly lower than that on buffer. The analysis of the percentage decrease of compressional modulus at 30 mN/m illustrates better the findings mentioned above (Fig. 2). A significant effect of the preservative on the morphology of the condensed phase formed at higher surface pressures was observed in BAM images for this system (*E.coli*) (Fig. 1). In contrast to the film on a buffer, in the presence of BCE the condensed phase is formed at higher surface pressure and the images evidence the less condensed (dark) regions in a more condensed (brighter) matrix (above 48 mN/m) However, the images obtained on a preservative solution are slightly brighter in the whole range of the film compression as compared to the monolayer on a buffer. In this case, it does not mean that the monolayer is more condensed. As it was mentioned before the BCE presence has fluidizing effect on the *E. coli* model membrane. The brightness of the images can be attributed to the BCE presence at the interface after the incorporation into the film (penetration experiment results are described in the next paragraph).

In the case of the films mimicking the *S. aureus* membrane in the presence of BCE both the shift of the isotherms to larger areas and the shift of the plateau region to higher surface pressures are well noticeable (Fig. S1, Fig. 2). The strongest differences in the shape and position of the isotherms appear at two the highest BCE concentrations. At these conditions also the collapse of the monolayer is observed at the values ca. 10 mN/m higher than the collapse of the remaining films. The percentage shift of the area at $\pi = 30$ mN/m and at BCE concentrations between 1 and 25 μg/mL is strong and linear, and some stabilization of the curve (Fig. 2) can be observed only at the highest preservative concentrations. At 10 mN/m the trends in the shift of the isotherms are similar to that found for *E. coli* membrane.

Considering the compressional modulus values, independently of BCE concentration *S. aureus* model membrane are in the condensed state. However, the C_s^{-1} vs π curves for the respective preservative concentrations differ from each other. Namely, the presence of BCE visibly affects the position of a minimum, the latter being attributable to phase transition. Moreover, the compressional modulus values strongly decrease with increasing concentration of BCE in the subphase. It can be concluded that the presence of the preservative hinders the formation of a more condensed state. The latter is well observable in BAM images. Namely, the collected images evidenced that the domains of the condensed phase are formed at higher surface pressures as compared to the film on a buffer. Additionally, the morphology of these domains differs from that for the film on a buffer, and finally the monolayer remains inhomogeneous up to ca. 40 mN/m which also is in contrast to the system on buffer. In comparison to the BAM images obtained on a buffer, the images taken on the highest BCE concentration are noticeably brighter and it might be a result of preservative incorporation into the lipid film. Additionally, they are also brighter than those for *E. coli* membrane, which may suggest stronger incorporation of BCE into *S. aureus* film.

Comparing the effect of BCE on both model systems it can be concluded that the studied preservative formulation causes stronger shift of the isotherm and stronger decrease of the compressional modulus in the case of *S. aureus* model system. Thus, the preservative exerts stronger fluidization of this membrane, which is also confirmed in BAM images.

In Fig. 3 and Fig. S2 the results of penetration experiments for both model systems are shown.

For *E. coli* membrane the penetration is stronger at lower surface pressure. The MIP and synergy parameter obtained from the experiments performed at different initial surface pressures at one preservative concentration (Fig. S2) are 43.7 mN/m and 0.81, respectively. Thus, the molecules in the studied formulation can insert into this model system even at very high surface pressures, while the positive value of the synergy parameter indicates that the binding to the monolayer is a favorable process [26]. However, it was found that at 10 mN/m the incorporation is visibly stronger at lower BCE concentration. Usually the

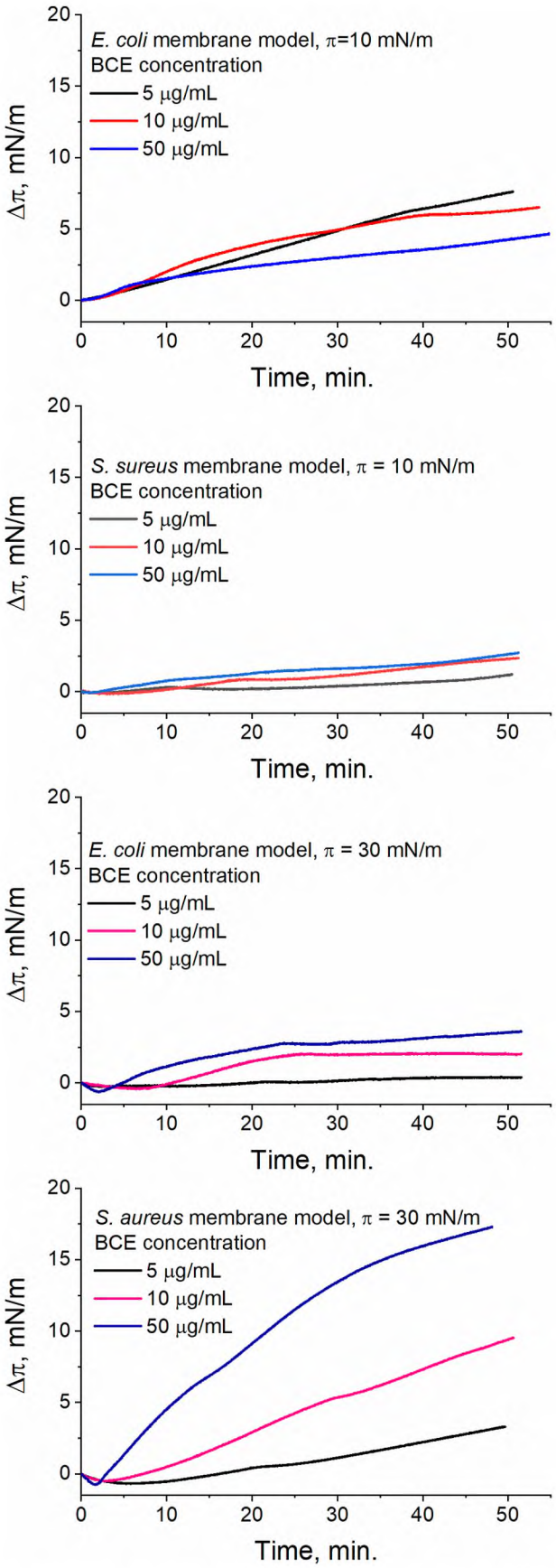

Fig. 3. The penetration of the components of BCE used in various concentrations (5; 10 and 50 μg/mL) into *E.coli* and *S. aureus* model systems at low (10 mN/m) and high (30 mN/m) surface pressure.

observed trend is that the penetration increases (or stabilizes) with the concentration of the membrane active molecule. The latter trend is obeyed at 30 mN/m for *E. coli* and both at low and high surface pressure for *S. aureus* membrane. However, for *S. aureus* membrane another unexpected feature is observed. Namely, the penetration increases with the surface pressure. This is well noticed in the plots presented in Fig. 3 and Fig. S2. Usually, the penetration decreases with the surface pressure since the condensation of the monolayer increasing with π makes the incorporation more difficult. However, in this case probably not the condensation of the film itself but its molecular organization favoring the insertion of molecules into the film plays a decisive role. This unusual behavior of this system penetrated by BCE does not allow us to determine MIP and synergy parameter values for *S. aureus* membrane. However, the obtained results evidence that the penetration occurs, and it is strong even at the membrane related surface pressure (30 mN/m).

3.2. The effect of BCE on the one-component films

Similarly to the mixed systems studied earlier, also the isotherms for one component lipid monolayers on a buffer and on the subphases containing various concentrations of BCE were recorded (Supplementary Materials, Figs. S3 and S4 – *E. coli* and *S. aureus* components respectively). At the same figures the values of C_S^{-1} *vs* π are presented. In Table 1 the characteristic parameters for one component monolayers are gathered.

The *E. coli* model consists of POPE, POPG and TOCL. The properties of the monolayers formed by these lipids are well-known and they were widely discussed before [e.g. 27]. In short, considering the parameters taken from the isotherms it can be concluded that the condensation of these films increases in the order: TOCL<POPG<POPE. In the course of the most tightly packed POPE film a phase transition at high surface pressures appears. The BAM images for TOCL and POPG monolayer reflect fully homogenous film therefore they are not presented herein. The pictures for POPE monolayer are shown in Fig. 4. As it is seen at the phase transition region the patches of the condensed phase are formed. With the increase of π the domains become bigger and above 45 mN/m they merge together and the monolayer is homogenous again.

Considering *S. aureus* model lipids both PG di-15:0 and TSCL are the lipids having only fully saturated chains, therefore they form the films more densely packed than their less saturated counterparts (POPG and TOCL, respectively). This is reflected in the parameters presented in Table 1. In the course of the curve for PG di-15:0 film at ca. 13 mN/m Liquid Expand (LE) - Liquid Condensed (LC) phase transition occurs. This transition is well observable in BAM images (Fig. 4). Namely, at ca. 13 mN/m small domains can be noticed. Their number and size increase up to the surface pressure of ca. 30 mN/m, where the domains merge and the monolayer is mostly homogenous, only small patches of a more condensed state can be observed.

The isotherm for TSCL film on a buffer rises up at ca. 130 Å^2/molecule and at 6 mN/m its course starts being very steep till ca. 45 mN/m where the collapse occurs. In BAM images, at large areas, the coexistence of gaseous and expanded phases can be noticed till $\pi \approx 1$ mN/m. Then, the regions of the gaseous phase disappear, and the pictures

Table 1
The properties of one component lipid monolayers.

	lift of area Å^2/molec.	Area$_{\pi = 30\ mN/m}$ Å^2/molec.	$C_{S30\ mN/m}^{-1}$ mN/m	C_{Smax}^{-1} mN/m	π at phase transition mN/m
POPE	92	57	115	260	35
POPG	120	64	90	100	–
TOCL	225	130	100	110	–
TSCL	130	85	310	310	–
PG di-15:0	115	46	230	275	13

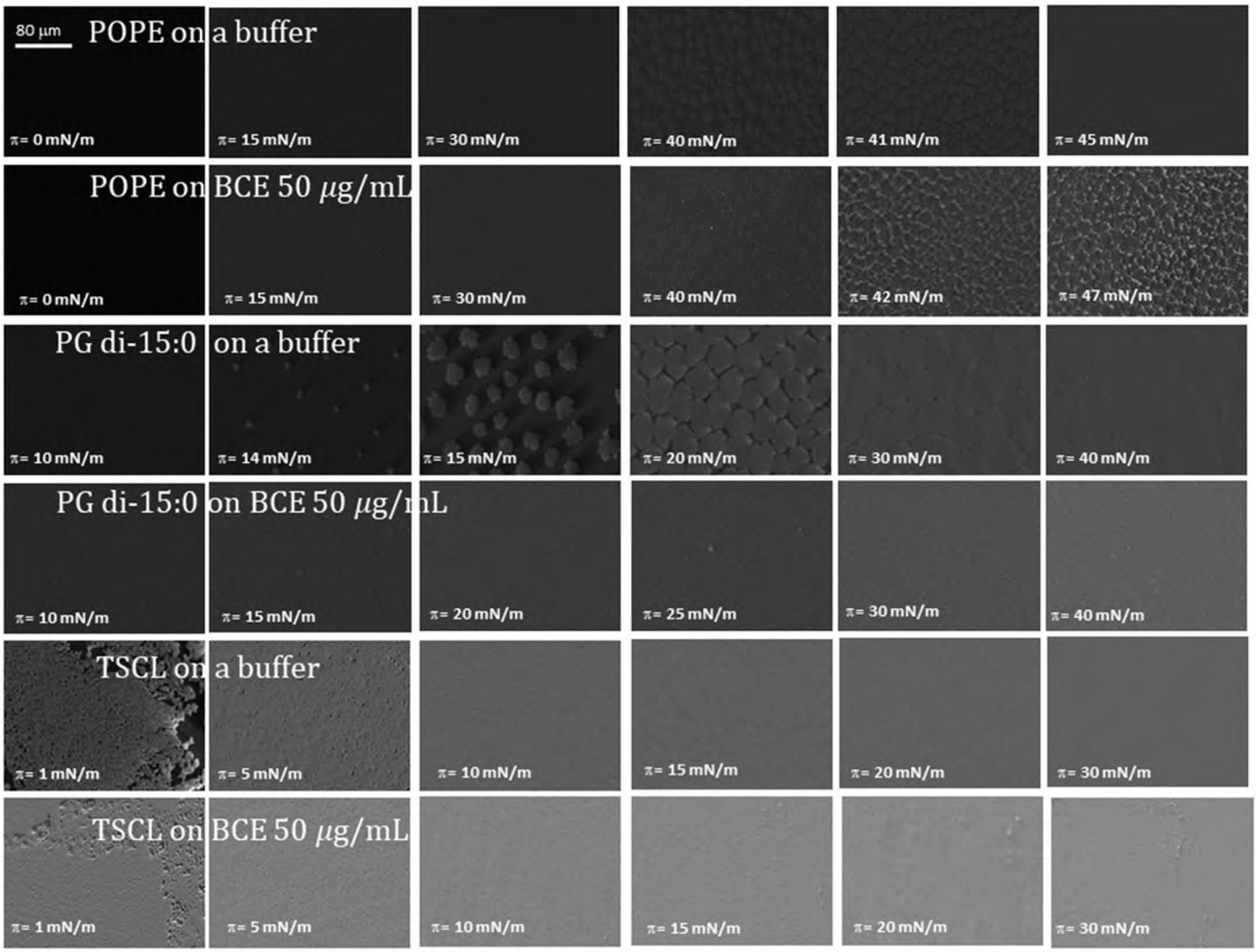

Fig. 4. BAM images for one component lipid monolayers on buffer and on the BCE solution.

become uniform and reflect the film in a liquid condensed state.

In the presence of BCE (Figs. S3 and S4) all the isotherms are shifted to the larger areas, and the phase transition surface pressure for POPE and PG di-15:0 monolayer is increased. This effect is especially pronounced for PG film. These findings prove that BCE decreases the condensation of these films, which is well manifested also in BAM images (Fig. 4). Namely comparing the images for POPE, PG di-15:0 and TSCL on buffer and on BCE solutions it can be seen that the preservative: i) hinders the formation of the condensed phase (the condensed phase is formed at higher surface pressures); ii) changes the morphology of the condensed domains and iii) shifts the transition surface pressure to higher values.

Deeper analysis of the effect of the studied formulation on the isotherms was performed based on the shift of the area presented in a percentage term in Fig. 5.

Comparing the results for one component lipid films it can be found that the effect of BCE on the isotherm position decreases in the order: PG di-15:0 > POPG > TOCL > TSCL > POPE. This means that the negatively charged lipids of similar polar head structure are more affected by the investigated formulation. However, there is no correlation when we compare the lipids differing only in the structure of a hydrophobic part of the molecule (PG di-15:0 vs POPG and TOCL vs TSCL). It seems that the latter confirms that the organization of the monolayer is important from the point of view of the effect of BCE. Moreover, it is evident that the films formed by POPG and TOCL being the component of *E. coli* model membrane are more affected by BCE than the mixed film (*E. coli* system). It is also clear that POPE as the major component of *E. coli* film regulates the effect of the preservative on the model system.

The influence of BCE on *S. aureus* membrane is intermediate between the effect on PG di-15:0 and TSCL. This relationship could be expected taking into account very similar proportion of both these lipids in the mixed system.

The penetration results for the studied monolayers are shown in Fig. 6. Firstly, the components of BCE formulation are inserted into all the investigated monolayers. However, for POPE film the incorporation occurs only at lower surface pressure. Secondly, at 30 mN/m the penetration into *E. coli* model system and into its individual component films (POPG and TOCL) is comparable. Thus, in this case the major membrane component (POPE) is not decisive from the point of view of membrane penetration. For *S. aureus* membrane at 10 mN/m the insertion into the mixed system and PG di-15:0 is comparable and much weaker than the insertion into TSCL. At 30 mN/m, in time, the penetration for both one component films and the model membrane becomes similar.

Comparing the incorporation of BCE components into the lipid films at low and high surface pressure it can be concluded that for *E. coli* lipids (POPE, POPG, TOCL) the higher the surface pressure the weaker the incorporation. However, for *S. aureus* lipids (PG di-15:0, TSCL) the penetration increases with the surface pressure. A similar trend was found for the mixed monolayer imitating *S. aureus* membrane.

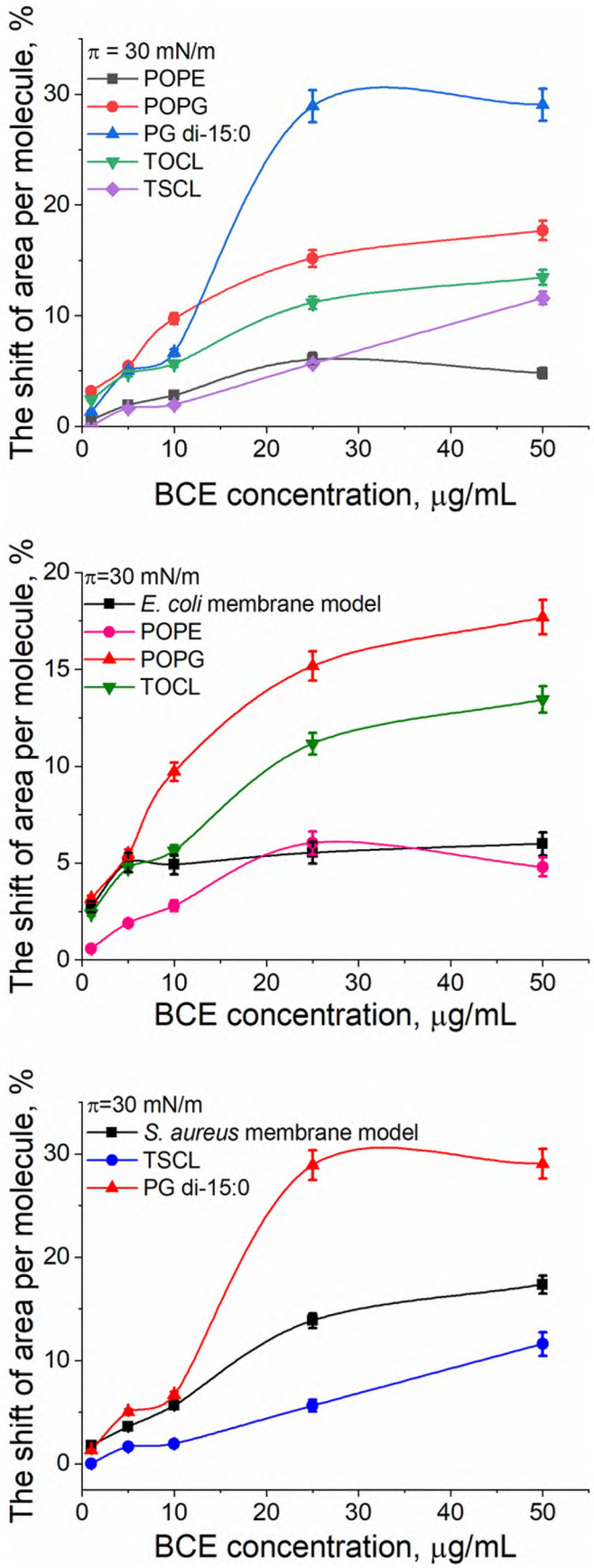

Fig. 5. The shift of the area per lipid values for the studied monolayers at 30 mN/m (in percent).

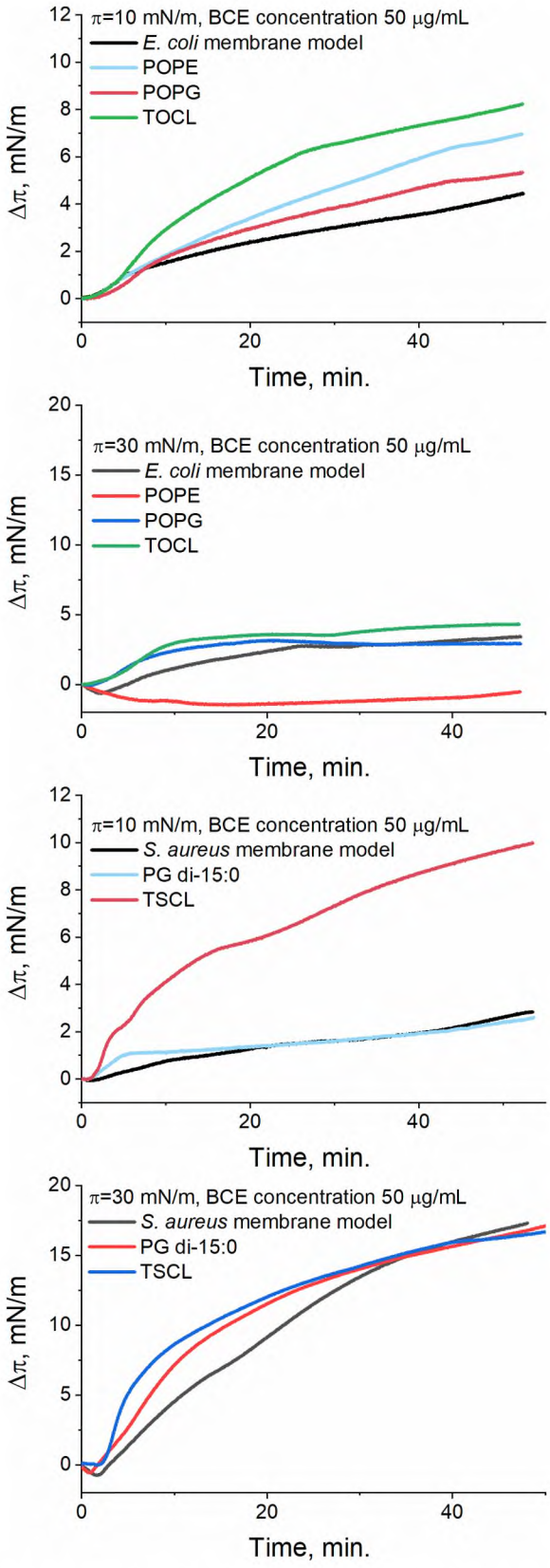

Fig. 6. The penetration of the components of BCE used in the concentrations 50 μg/mL into the one component lipid monolayers at low (10 mN/m) and high (30 mN/m) surface pressure. For the comparison the results for *E.coli* and *S. aureus* model systems are also presented.

Stronger penetration of BCE components into PG di-15:0 and TSCL films as compared to the monolayers on buffer reflects also in BAM images. Namely, the BAM images taken on BCE containing subphase are brighter than the pictures on a buffer. As results from penetration experiments the BCE penetration into TSCL films is much stronger than into PG di-15:0 at $\pi = 10$ mN/m and comparable at $\pi = 30$ mN/m. In the consequence, the images TSCL, in the whole surface pressure range are much brighter than taken on a buffer, while in the case of PG di-15:0 significant changes in the picture's brightness are observed above $\pi = 25$ mN/m.

3.3. *In vitro* tests on bacteria strains

In Table 2 the results of *in vitro* experiments on bacteria strains as the percentage of bacterial growth in the presence of BCE in relation to the control growth without the addition of the substance are presented.

The MIC values for *E. coli*, *S. aureus*, and *S. enterica* and *P. aeruginosa* were 1 mg/mL, while for *E. faecalis*: 10 mg/mL. BCE exhibited bactericidal activity against all tested bacteria and MBC values were determined. Lower bactericidal concentrations (MBC = 1 mg/mL) were observed for *E. coli*, *S. aureus*, and *S. enterica*. For *E. faecalis* and *P. aeruginosa* MBC values were ten times higher (MBC = 10 mg/mL). However, for *P. aeruginosa* the concentration of 1 mg/mL completely inhibited the growth of bacteria while the concentration 0.5 mg/mL reduced growth to 14% compared to growth control. *E. faecalis* in the presence of BCE in concentrations up to 1 mg/mL showed growth comparable to control without the addition of the tested preservative and no antibacterial effect was observed. For *E. coli*, *S. aureus*, *S. enterica* the concentration half that of MBC (0.5 mg/mL) resulted in a weakening of the growth to 77%, 78% and 52%, respectively. BCE concentrations ranging from 0.001 mg/mL to 0.125 mg/mL showed little or no antibacterial effect against tested strains. The percentage of bacterial growth in the presence of tested preservative in comparison to control growth exceeded 90%.

BCE is the most active against Gram-negative *S. enterica* and *P. aeruginosa*. The effect of BCE on *S. aureus* and *E. coli* is comparable but in concentrations $\leq$0.5 mg/mL is weaker than against *S. enterica* and *P. aeruginosa*. *E. faecalis* was the most resistant species to tested compound. Only the highest concentrations of preservative had an antibacterial (bactericidal) effect.

4. Discussion

Summarizing the most important results of performed experiments two major conclusions can be drawn. Firstly: the studied preservative formulation affects organization of both *E. coli* and *S. aureus* model membranes by decreasing their condensation. And secondly: the results of *in vitro* tests confirm very similar antibacterial activity of BCE formulation against *E. coli* and *S. aureus*. However, for a more detailed analysis of the collected results it is necessary to relate them to literature data.

The blackcurrant extract, which is a base of the investigated herein preservative formulation, is known from a wide antimicrobial effect against various pathogens [22,28–30]. However, the magnitude of its activity is determined by various factors related to the extract's origin. For example, extract from blackcurrant berries was found to be of higher antibacterial activity against *E. coli* (MIC = 55.82 μg/mL) as compared to S. *aureus* (MIC = 125.05 μg/mL) [22]. However, the determined MIC values depend strongly on blackcurrant cultivars and in the test on *E. coli* they were in the range of 42.7–65.1 μg/mL, while in the studies on *S. aureus* 106–148 μg/mL [22,28,29]. The important factor is also the manner of cultivation. Namely, depending on the soil management system (bare fallow, sawdust mulch and black plastic mulch) MIC values for blackcurrant extract were in the range of 38.2–56.9 μg/mL for *E. coli* and 101–121 μg/mL for S. *aureus*. As it was evidenced, the foregoing factors determine the composition of extract and in this way modulate their activity against microorganisms. For example, blackcurrant berries are characterized by lower MIC values against microorganisms than leaves, which was attributed to a higher level of total phenolic compound flavonoids, condensed tannins and gallotanninsin fruit extract in berries as compared to leaves [22].

Moreover, by comparing the results of tests against various bacteria species obtained in the same experimental procedure it is practically impossible to indicate, which bacteria: Gram-positive or Gram-negative are more susceptible to this extract. The MIC values obtained in the tests against *E. coli* were the lowest among all the tested species (55.82 μg/mL). However, the results reported for other Gram-negative species were rather similar among themselves and they suggest lower susceptibility of these bacteria to extract than *E. coli* (e.g. *Klebsiella pneumoniae* – ca. 123 μg/mL, *Proteus vulgaris* – 121 μg/mL, *Proteus mirabilis* – 130 μg/mL). Additionally, the mentioned above MIC values obtained in tests on Gram-negative bacteria were also very similar to the values collected in the studies on Gram-positive bacteria (*S. aureus* – 125.05 μg/mL, *Bacillus subtilis* – 133.83 μg/mL) [22].

Considering the results of our *in vitro* test, the obtained MIC and MBC values confirm that the activity of the investigated BCE formulation is similar against *E. coli* and *S. aureus*. This is also in agreement with the information reported by the producer [13]. However, also our results do not provide information on the correlation between the structure and the morphology of bacteria cell (Gram-positive *vs* Gram-negative species) and BCE activity. This is very important finding taking into account the mechanism(s) of action of blackcurrant extract mentioned in literature [30–32]. The first barrier between the environment and the bacteria cell is membrane. Gram-positive and Gram-negative bacteria differ in the structures of membranes surrounding the cells. In the studies on the effect of the phenolic extract from blackcurrant was evidenced that it is able to release of lipopolysaccharide (LPS) molecules from the outer layer of Gram-negative bacteria membrane [32]. This leads to disintegration the outer bacteria membrane and increases sensitivity of bacteria to the active molecules. The ability of blackcurrant phenolic extract

Table 2
Antibacterial activity of BCE.

The percentage of bacterial growth in the presence of tested substance in relation to the control growth without the addition of the substance					
BCE concentration	*Escherichia coli* ATCC 25922	*Enterococcus faecalis* ATCC 29212	*Staphylococcus aureus* ATCC 29213	*Salmonella enterica* BAA-664	*Pseudomonas aeruginosa* ATCC 9027
10 mg/mL	1%	0%	0%	0%	0%
1 mg/mL	0%	100%	0%	6%	0%
0.5 mg/mL	77%	100%	78%	52%	14%
0.25 mg/mL	93%	100%	86%	77%	81%
0.125 mg/mL	94%	100%	96%	95%	99%
0.1 mg/mL	98%	100%	93%	95%	99%
0.05 mg/mL	92%	100%	100%	97%	100%
0.01 mg/mL	96%	100%	100%	100%	99%
0.005 mg/mL	95%	100%	100%	98%	100%
0.001 mg/mL	95%	100%	100%	99%	98%

to LPS liberation was attributed to the presence of weak organic acids (e.g. citric acid) in the studied samples, which increases permeabilization of outer Gram-negative bacteria membrane [32]. This may indicate a stronger effect of blackcurrant extract on Gram-negative species. However, the overcoming the outer membrane barrier and its deformation are not the only sites of the activity of polyphenols [33,34]. It has been shown that these compounds disrupt bacteria cellular membrane (inner membrane) and cause the loss of chemiosmotic control, lead to morphological changes and release of vitality important molecules from cellular environment. Thus, not only the outer membrane (namely the structure differing Gram-positive and Gram-negative species) but also the inner lipid bilayer is important from the point of view of antibacterial effect of polyphenols and the other components of extract.

The analysis of flavonoid profile of the studied herein BCE (Table S1, Supplementary Materials) evidenced that the major compounds are: epigallocatechin, (−)-epigallocatechin 3-*O*-gallate and (−)-epicatechin 3-*O*-gallate, but also catechin, epicatechin and other flavonoids were detected. There is a number of evidences in literature that these compounds have antibacterial properties [e.g. 35–38]. Moreover, they are also active at the membrane level, but the magnitude of flavonoid/membrane interactions depends on the molecular structure of the studied compound. However, the membrane activity of flavonoids and their ability to alter these cellular structures were indicated as the target important from the point of view of antimicrobial activity of these compounds [35–38]. In addition, it should be emphasized that the studied BCE formulation is a complex mixture of compounds. Its effect on membrane, interactions with membrane constituents and antimicrobial activity are not determined only by the content of individual ingredients, but also by the interactions between them, which may strengthen or weaken the effect of the mixture compared to the effect of individual ingredients (synergism/antagonism).

The results presented herein for model lipid membrane systems provide an insight into the interactions of BCE formulation components with the lipids. The results of experiments performed on model lipid bacteria membranes evidenced that the studied preservative affects organization of both *E. coli* and *S. aureus* model systems. Namely, in the presence of BCE both models become less condensed and their morphology undergoes alterations to be more fluid. As evidenced in the penetration experiments the components of BCE, which is a mixture of compounds, are inserted into the monolayers. However, for *E. coli* system the effect of BCE on the monolayer condensation does not increase with BCE concentration and additionally at 10 mN/m it is stronger at lower BCE concentration. This allows one to suggest that there is a limit of the molecules, which can be accommodated within this lipid environment and above some concentration they cannot be inserted into the membrane. As proved the experiments on erythrocytes, model erythrocyte membrane and erythrocyte lipids [20,39], the components of blackcurrant fruit and leaf extract and polyphenolic compounds from selected fruits, bind to erythrocyte membrane surface and change the packing of the lipid molecules in the region of polar heads. However, they do not penetrate into the hydrophobic region and they do not affect fluidity in this region of membrane.

The results collected in our experiments proved that the effect of BCE is stronger on the system imitating *S. aureus* membrane as compared to *E. coli* film. Moreover, it can be suggested that not the condensation of the monolayer itself but rather the organization of the film determines the affinity of BCE components to the lipid system. Namely, as it was found for *S. aureus* membrane the insertion of the components of BCE formulation increases with the surface pressure. The latter can be surprising since the increasing (with π) condensation of the film usually prevents from the penetration of the active molecules. In the context of the membrane organization, the packing defects being the areas of a decreased density of lipid molecules, should be considered. In flat membranes, they are formed due to the presence of cone-shaped lipids with a small head group (for example PE, cardiolipin) and their formation is also facilitated by the presence of unsaturated chain(s) in the lipid molecules. The lipid packing defects make the binding of molecules to the membrane easier which was evidenced for many studied systems [40,42–44,NaN–NaN,NaN].

It is clear that the organization of the monolayer is determined by its composition. The investigations performed on one component films evidenced that the components of BCE more strongly affect the negatively charged lipids and among them phospahtidylglyceroles. However, for both model systems the proportion of particular components does not determine the effect of BCE on the mixed system. Namely, for *E. coli* membrane, which is dominated by POPE molecules, the penetration is similar to the penetration into the remaining minor components (POPG and TOCL), but not to major POPE. For *S. aureus* membrane the penetration for both one component films and the model membrane is similar.

The fact that the components of blackcurrant extract destroy the structure of the outer membrane of Gram-negative bacteria (e.g. *E. coli*) [32] does not reflect in a stronger antimicrobial effect of BCE formulation on this type of bacteria. Similarly, the stronger effect of BCE on the model membrane of Gram-positive bacteria (*S. aureus*) is not reflected in stronger antibacterial activity of the preservative to Gram - positive species. Therefore, it can be concluded that similar activity of BCE against *E. coli* and *S. aureus* found in our *in vitro* test may suggest that the mechanism of action of this preservative on Gram - negative and Gram - positive species is different.

Summarizing, the studied BCE formulation possess antibacterial activity however, its magnitude is a sum of various factors. Namely, the activity depends on the morphology of bacteria cell and on the lipids forming bacteria membrane.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2023.109806.

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