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An influence of molecular weight, deacetylation degree of chitosan xerogels on their antimicrobial activity and cytotoxicity. Comparison of chitosan materials obtained using lactic acid and CO_2 saturation

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ABSTRACT

This paper presents a comparison of the antimicrobial activity and cytotoxicity against L929 cells of chitosan xerogels prepared by dissolving the polymer in a solution of lactic acid (LA) or carbonic acid (CO_2) and then freeze-drying. There was no simple relationship between the antimicrobial activity and cytotoxicity of the samples obtained using both techniques (LA and CO_2). Chitosan materials obtained by the LA method in a 1:1 dilution were characterized by the highest cytotoxicity against L929 cells (~20%). For the same diluted samples prepared using the CO_2 saturation method, the viability of L929 cells was approximately 2.5 times greater. Some of the tested chitosan materials obtained by the innovative method were characterized by significantly lower antimicrobial activity, for example, reduction of *E. coli* bacteria for MMW-LA and MMW-CO_2 samples by 6.00 and 0.75 logarithmic order, respectively. This clearly indicates that in many applications, the presence of the acid necessary to dissolve chitosan is responsible for the antimicrobial activity of the polymer solution and its products.

1. Introduction

Chitosan is a natural polysaccharide with a positive charge, and is a derivative of chitin. It consists of the monomers N-acetyl-D-glucosamine and D-glucosamine linked by β-1,4-glycosidic bonds [1]. Chitosan occurs sporadic in the biosphere, therefore this polymer is obtained from chitin, which is one of the main components of the exoskeletons of arthropods such as shrimp, crabs, lobsters and arachnids [2]. The most common method for obtaining chitosan is the deacetylation of chitin, which is a two-stage nucleophilic substitution reaction that takes place in an alkaline solution. Depending on the origin of chitin and parameters of the deacetylation process, chitosan can be obtained with different molecular weights (MW) and degrees of deacetylation (DD). These parameters affect the physicochemical and biological properties of biopolymer-based materials based on this biopolymer [1–4].

The properties of chitosan, such as biocompatibility, biodegradability, non-toxicity, antimicrobial activity, hemostatic properties, and ability to accelerate wound healing, make it an object of research and application in designing dressing materials [5], scaffolds [6], and carriers for the controlled release of drugs and other biologically active pathways [7]. Chitosan can also be used as a component of food packaging or as a food additive, inhibiting the development of microorganisms and thus prolonging the freshness of the product u [8].

One of the most significant features of the potential action of chitosan is the antimicrobial activity of the polymer, which is the result of many different factors, including DD and MW [9]. Zheng and Zhu [9] and Tavaria et al. [10] reported an increase in the antimicrobial activity of chitosan against Staph. aureus bacteria with increasing MW [9,10]. Despite the relatively well-determined effect of the MW of chitosan on its antimicrobial activity against Gram-positive bacteria, many inconsistent results of its activity against Gram-negative bacteria can be found [11]. Some studies on *E. coli* and *Salmonella* have indicated an increase

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in antimicrobial activity with decreasing MW [9,12,13]. Other results for the same class of bacteria showed an increase in performance as the MW increased to the determined value and then decreased with increasing MW, or different performances for different MW and different types of bacteria of the same class ([14,15], n.d.). The justification for these differences is the method of processing the chitosan material, the pH of the solution, or the DD [16]. In addition, the source of chitin from which chitosan was produced, as well as the method of processing, play an important role in the antimicrobial activity of chitosan materials [17–19].

Several models have suggested that the antimicrobial activity of chitosan is due to its cationic nature. The electrostatic interaction between positively charged polymers and negatively charged microbial cell membranes is predicted to be responsible for cellular lysis, and is assumed to be the main antimicrobial mechanism [20].

The second mechanism is based on the binding of chitosan to bacterial DNA, which leads to the inhibition of protein synthesis as a result of chitosan penetration inside the cells of microorganisms. This model assumes that chitosan can pass through the walls and/or membranes of bacterial cells, as confirmed by scanning techniques [2].

The third mechanism of the antimicrobial activity of chitosan is related to its ability to chelate metal ions. It consists of binding the polymer to nutrients necessary for the growth of microorganisms [20]. This mechanism applies in particular to the alkaline environment because the amino groups are in the unprotonated state, and the electron pair present on the nitrogen of these groups can interact with cations [21,22].

Chitosan is generally considered safe, which has been confirmed by numerous studies presenting in vitro tests carried out on cell lines [23–25], and in vivo tests carried out on model organisms living ([26, 27], 1997b). Despite the proven harmlessness of chitosan, some studies have indicated the possible cytotoxicity of materials produced through its participation [28,29]. It is assumed that the cytotoxicity may depend indirectly on the method of processing and the origin of chitin because the influence of the DD and the MW of chitosan on the cytotoxicity phenomenon was observed. The lower molecular weight (LMW) chitosan showed less cell-damaging effects than its higher molecular weight (HMW) counterpart. This phenomenon is probably correlated with the surface charge density distribution and amount of free amino groups. Their greater amount in the case of chitosan with a higher MW results in stronger electrostatic interactions between the chitosan chains, and thus the formation of a more developed polycation molecule that attaches more easily to the cell [30,31].

There is a lot of information in the scientific literature on the biological properties of chitosan, but finding an answer to the relationship between the cytotoxic effect and antimicrobial activity of chitosan materials is practically impossible. The biological properties of these materials are subject to high variability resulting from the source of the polymer, method of extraction and processing, and different conditions of freezing and freeze-drying to obtain porous materials [32–34]. This paper presents the change in antimicrobial activity and cytotoxicity of xerogel chitosan materials resulting from the difference in the DD and MW of the polymer, and the technique of its dissolution. The influence of the use of an innovative procedure for obtaining chitosan xerogel materials consisting of dissolving the polymer by saturation of its suspension with carbon dioxide (CO_2) [35] on the above parameters is presented. To date, no attempt has been made in the scientific literature to assess these relationships, taking into account the above variables.

According to the research hypothesis, the lack of acid residues in xerogel chitosan materials obtained by the innovative method of saturation with gaseous CO_2 will allow the actual assessment of the antimicrobial activity of chitosans resulting from the structures of the polymer itself and differences in MW and DD. In addition to maintaining this activity, the prepared materials will be characterized by lower cytotoxicity in relation to the L929 model cell line in comparison to xerogels created by classical chitosan dissolution in acid, freezing and lyophilization.

Standard methods of producing chitosan xerogels involve predissolving the polymer in an acid solution, freezing the solution and freeze-drying it. During this operation, water is removed from the material by sublimation, and acid anhydrides are formed, which remain in the material in an amount depending on its volatility and boiling point. After contact with water, acid is formed again in the chitosan xerogel, i.e. hydrogen cations and anions of the acid residues used to dissolve the polymer are present. In the CO_2 saturation method, chitosan is initially dissolved in a solution of hydroxyacetic acid (hydrogen cations, hydroxyacetic anions), then precipitated with sodium hydroxide, as a result of which chitosan is precipitated in a microxystalline form to produce sodium hydroxyacetate and water as a result of reaction. The precipitate is washed thoroughly until the pH is neutral (removal of sodium cations and hydroxyacetic anions). In the last stage, the chitosan precipitate is suspended in distilled water and saturated with carbon dioxide gas, which acidifies the environment (production of weak carbonic acid), which dissolves the polymer. Carbonic acid is a weak acid that dissociates only slightly and is unstable, decomposing fast into carbon dioxide and water, which means that the chitosan hydrogel ready for freeze drying does not contain any additional compounds that would affect the biological properties of the chitosan xerogels created.

2. Experimental section

2.1. Materials

Chitosan polymers with low (20–300 cps, DD ≥ 75%, Cat. No.:102473649, LOT: BCCG5629), medium (200–800 cps, DD ≥ 75%, Cat. No.:102466463, LOT: BCCG9377), high (800–2000 cps, DD ≥ 75%, Cat. No.:102515456, LOT: BCCH4876) MW, chitosan isolated from crab (Cat. No.:101167160, LOT: BCBH3811V) and shrimp shells (DD ≥ 75%, Cat. No.:1003507957, LOT: SLCP5257),phosphate buffer saline (PBS, Cat. No.:1002795530, LOT: SLBZ3711), Tryptic Soy Agar (TSA, Cat. No.:1.05458.0500, LOT: VM1009758 216), Tryptic Soy Broth (TSB, Cat. No.:1.05459.0500, LOT: VM899959 942), Lactic acid (LA; Cat. No.:1003429706, LOT:SHBP4889), hydroxyacetic acid (Cat. No.:102527954, LOT: STBK8247) were purchased from Merck. Chitosan with MW of 150 kDa (Cat. No.: 22741, LOT: 407568/1) was purchased form Fluka.Peptone K (Cat. No.:S-0011, LOT: S011130306) was purchased from BTL Sp. Z o.o.(Poland). Acetic acid (Cat. No.:568760114, LOT:1024/04/19), hydrochloric acid (Cat. No.: 115752837 LOT:210305322), and sodium hydroxide (Cat. No.: 115752837 LOT:210305322) were purchased from Avantor Performance Materials Poland S.A. (Gliwice, Poland). Viscosity standards in the form of mineral oils for viscosimeter calibration were purchased from IKA (10 mPas Cat. No.: 25000398, LOT: 280038/1, 100 mPas (Cat. No.: 25000434, LOT: 07040, 1000 mPas (Cat. No.: 25000436, LOT: 07119) (Warsaw, Poland) Viscosimeter standards – poly(ethylene glycol) with MW of 200 kDa (Cat. No.: 102511497 LOT:BCCG7893, 400 kDa Cat. No.: 102550289 LOT:BCCH5871, 1000 kDa (Cat. No.: 8.07488.1000 LOT:S8283588 247. The CO_2 used to saturate the chitosan precipitate was obtained from Linde Gaz Polska Sp. Z o. o. (Gdansk, Poland). For microbiological tests, the following bacterial species were used: Gram-negative *Escherichia coli* (ATCC 25922) and Gram-positive *Staphylococcus aureus* (ATCC 29213) from the Polish Collection of Microorganisms, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences (Wrocław, Poland). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Cat. No.:1003404689, LOT: MKCR748), medium, antibiotics, and supplements necessary for cell culture were obtained from Merck (Germany). MilliQ water was used to prepare all the aqueous solutions (Milli-Q® IQ 7005 Water Purification System, Millipore, USA). All other reagents were of analytical grade or higher.

2.2. Chitosan characterization

2.2.1. Degree of deacetylation

The DD of chitosan was determined using the potentiometric titration method [36]. The titration solution was prepared by dissolving 0.25 g of chitosan in 10 mL of 0.3 M hydrochloric acid and filled up to 200 mL). It was titrated with a 1 M solution of sodium hydroxide, which allowed the pH vs. amount of added NaOH titration curve to be plotted. Then, using MatLab software (R2022a, The MathWorks, Inc.), the inflection points of the titration curve were determined. The mass of used chitosan (M), value of measured pH for the first inflection point (x), and value for the second inflection point (y) were applied in equation (1) to calculate the percentage of chitosan-free amino groups (%NH_2).

$$\%\, \boldsymbol{NH_2} = \mathbf{16.1}\,(\boldsymbol{y}-\boldsymbol{x})/\boldsymbol{M} \quad (1)$$

2.2.2. Molecular weight

The MW of the chitosan polymers was determined by an indirect method using dynamic viscosity measurements with a Brookfield Digital Model DVIII Ultra viscometer equipped with a temperature-controlled bath (Middleboro, MA, USA). The viscometer was calibrated using viscosity standards in the form of mineral oils with viscosities of 10 mPas, 100 mPas, and 12500 mPas (IKA, Warsaw, Poland) to measure spindles SC4-18, SC4-27, and SC4-25, respectively, whose measuring range corresponds to the viscosity of the measured chitosan solutions. Then, the viscosity dependence of the standard glycol solutions and chitosan solution with a defined MW (150 kDa; 1% m/v solutions in 1% v/v acetic acid) as a function of shear rate at 25 °C was determined. In order to determine the MW of chitosans, a 1% solution (m/v) in 1% acetic acid (v/v) was prepared from each of them, and their viscosities were measured at 25 °C using an appropriate measuring spindle adjusted based on the generated strain as a result of shear stresses between the liquid layers during the measurement. For each of the measuring spindles, the relationship between MW and dynamic viscosity was used to determine the MW of chitosans with unknown MW. All rheological measurements were performed in triplicate.

2.3. Chitosan xerogels preparation

Xerogel materials based on chitosan were prepared using two dissolution methods: the classic method using a 0.1 M LA solution [37] and the method of CO_2 saturation, an aqueous suspension of chitosan in the microcrystalline form [35]. The chitosan concentration in the solution was 1% v/v. The solutions in LA were prepared by systematically pouring chitosan powder into a LA solution during mechanical mixing at a speed of 300 RPM (RA 2020, Heidolph Instruments GmbH & Co. KG, Kelheim, Germany) and stirred until the polymer was completely dissolved (approximately 1 h). The chitosan solution was prepared by CO_2 saturation, as follows: In the first step, a 1,5% chitosan solution in 0.1 M hydroxyacetic acid was obtained by indirect dissolution of the polymer in a proper acid solution during mechanical stirring at a speed of 300 RPM (RA 2020, Heidolph Instruments GmbH & Co. KG, Kelheim, Germany). Then, during mixing 0.5 M solution of sodium hydroxide solution was added until a pH value in the range of 9–10 was reached. This was equivalent to the complete precipitation of chitosan in the microcrystalline form. The precipitated chitosan was filtered using a seepage kit under reduced pressure and washed five times with distilled water. Finally, the precipitated chitosan was weighed and suspended in distilled water to obtain a solution of 1% relative to the dry matter of the polymer. The suspension was homogenized at 10000 RPM for 3 min (Silent Crusher M, Heidolph Instruments GmbH & Co. KG, Kelheim, Germany), and then saturated with CO_2 with simultaneous mechanical mixing using a hollow shaft stirrer for gas saturation (BIOMIX BMX-10, Gdansk, Poland) until completely dissolved. The obtained solutions were poured into flat forms of 10 × 20 cm size to a height of 5 mm, frozen at −80 °C, and then freeze-dryed (Pressure: 0.94 mbar, Condenser temperature: −80 °C, Sample shelf temperature: 50 °C). Before use in the tests, the samples were stored in a dry, tight package under cool conditions.

2.4. Solubility test with visualization

To compare the behavior of chitosans xerogel immersed in water, 0.5 g was suspended in 50 mL of distilled water for 24h at 37 °C. Subsequently, the samples were imaged using the TOP Show 3D automatic rotation system (Wroclaw, Poland).

2.5. Antimicrobial activity

The antimicrobial properties of the xerogel materials were evaluated according to the quantitative ASTM E2149 method with slight modifications using *E. coli* and *S. aureus* species [38]. Colonies of bacteria were first subcultured in TSB for 24 h at 37 °C. Then, from cultured bacteria test medium in TSB were prepared by adjusting the number of bacterial cells in the range 1.0–5.0 × 10^7 CFU/mL with a spectrophotometer, measuring the absorbance at 600 nm (optical density 0.1). The change in absorbance of a bacterial culture is proportional to the number of bacteria present in the sample. This is a relationship that describes how absorbance (A) changes as a function of the number of bacterial cells in the sample. This relationship can be described by the general equation: A (Absorbance) = ε absorption coefficient) *b (length of the light path through the sample) *c (concentration of absorbing substances in the sample). The more bacterial cells there are, the more light they absorb, leading to a higher absorbance value. The value of the absorption coefficient (ε) is specific to a given type of bacteria and the wavelength of light used for measurement.The materials for the study were prepared by cutting squares with a side of 4 cm from the chitosan xerogels. Polyethylene foil of the same dimensions was used as the control sample, which showed no antimicrobial activity. The cut materials were sterilized with UV radiation for 30 min on each side. Then, 0.4 mL of diluted bacterial inoculum was applied to the surface of the samples. Each square of the inoculated surface was covered with a sterile polyethylene film with the same dimensions to ensure contact of the cell suspension with the material on the surface of 16 cm^2. After 24 h incubation at 37 °C, samples were placed separately in 10 mL of PBS solution and vortexed intensively for 25 s. Next ten-fold serial dilutions were prepared, then seeded on TSA plates, and incubated for 48 h at 37 °C. After incubation, only plates containing 30 CFU–300 CFU were counted. When no colonies were recovered in non dilluted sample, the number of bacteria was recorded as "10." The viable count of bacteria (CFU/mL) was recorded using the following formula:

$$V_C = N \bullet D \quad (2)$$

where Vc is the bacterial concentration in colony forming units per mL (CFU/mL), N is the average value in colony forming units (CFU) from Petri dishes, and D is the dilution factor from the counted plates. The antimicrobial activity on a logarithmic scale was calculated using the following formula:

$$\mathrm{R} = \log(B/A) \quad (6)$$

where A is the average of the number of viable cells on the test sample after 24 h incubation at 37 °C (CFU/mL) and B is the average of the number of viable cells in the control sample after 24 h incubation at 37 °C (CFU/mL). A percentage reduction of bacteria on logarithmic (R) scale equal to 1, 2, and 3 corresponded to a reduction of 90%, 99%, and 99.9%, respectively.

2.6. Cytotoxicity

2.6.1. Cell culture

Adult mouse fibroblast L929 cells were purchased from the American

Type Culture Collection (ATCC, Manassas, VA, USA) and tested negative for mycoplasma using a Universal Mycoplasma Detection Kit (ATCC, Manassas, VA, USA). The L929 cell line was cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest, Nuaille, France), 100 μg/mL streptomycin, and 100 U/mL penicillin. Cells were incubated at 37 °C in a 5% CO_2 atmosphere. All experiments were performed using cells in the exponential phase of growth.

2.6.2. Cell viability and morphology assessment

To estimate the in vitro cytotoxicity of the extracts, the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was used, according to ISO 10993–5:2009(E). Briefly, L929 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and 100 μL of culture medium (blank) was dispensed into the peripheral wells. After 24h of incubation at 37 °C in a 5% CO_2 atmosphere, the culture medium was removed and replaced with either fresh medium (positive control and blank) or fresh medium containing samples (1:1 v/v, 1:5 v/v, and 1:9 v/v of chitosan extract). Samples for cytotoxicity studies were prepared by suspending 0.5 g of the chitosan xerogel in 50 mL of distilled water for 24h at 37 °C. After this time, the liquid part of the sample (extract) was passed through a 0.22 μm filter. Following 24h incubation, images of cells were taken using a 20× objective in an OLYMPUS I×83 inverted microscope with an XC 50 camera and cellSens Dimension software. The culture medium was then removed, and 50 μL of the MTT solution (1 mg/mL in medium without supplements and phenol red) was added to each well and incubated for 2h at 37 °C in a 5% CO_2 atmosphere. Next, the MTT solution was removed, and the formazan crystals were dissolved in 100 μL isopropanol and shaken for 10 min. Absorbance was measured at 540 nm using a microplate reader (iMarkTM, Bio-Rad, Hercules, CA, USA). The results were obtained from four independent experiments (n = 4).

2.7. Statistical analysis

STATISTICA software (StatSoft, Inc., Tulsa, OK, USA) was used for all the analyses. Statistical significance was set at $P < 0.05$. All data reported are based on the means of four replicates (n = 4). Experimental results are expressed as mean ± standard deviation (SD). Student's t-test and one-way analysis of variance (ANOVA) were applied. The Mann-Whitney *U* test was used to analyze the differences between the results of the cytotoxicity assay for LA and CO2—obtained materials (*$p < 0.05$).

3. Results and discussion

3.1. Chitosan characteristic parameters

Five different chitosan polymers were used to evaluate the relationship between the antimicrobial properties and cytotoxicity of the materials prepared using two different methods. Table 1 shows the results of the DD measurements and their MW.

The indirect measurement of the MW by viscometry confirmed the information provided by the chitosan manufacturer. The values for low molecular weight (LMW), medium molecular weight (MMW), and high molecular weight chitosan (HMW) are consistent with literature data, for which the MW range are 50–150 kDa, 150–500 kDa and 500–2000 kDa, respectively [37,39,40]. Data from the specification of chitosan from shrimp shells and crab shells indicate that these are HMW polymers, which is also confirmed by our results. In addition, the manufacturer described chitosan from crab shells as highly viscous. This is consistent with the results, as the mass of chitosan was as high as 4000 kDa. The highest measured MW of chitosan reported in literature is approximately 10 million dalton [41]. However, the MW of chitosan can vary depending on its source, method of preparation, and DD. The LMW, MMW and HMW chitosans do not differ significantly in the DD. Significant differences were observed only for chitosans with HMW (Table 1).

Table 1
Description of the tested chitosan samples, their DD and MW (n = 3, $p < 0.05$).

Seller chitosan name	DD [%]	MW [kDa]	Given symbol
Low molecular weight	75.7 ± 5.7[a]	89 ± 4[a]	LMW
Medium molecular weight	81.7 ± 4.8[a]	280 ± 10[b]	MMW
High molecular weight	78.8 ± 1.5[a]	591 ± 26[c]	HMW/H79
From shrimp shells[a]	66.2 ± 3.1[c]	545 ± 22[c]	HMW/H66
From crab shells[a]	83.2 ± 4.6[b]	4000 ± 142[d]	HMW/H83

[a] High molecular weight chitosan.

Therefore, the results were used to determine the variability of the biological properties of chitosan materials (antimicrobial activity and cytotoxicity) depending on the MW with the same DD (LMW, MMW, HMW) and the DD with the same HMW (H66, H79, H83). Chitosan materials were obtained by the classical method using LA dissolved in water (Fig. 1. B). This indicates that their preparation method, which includes a lyophilization step, retains the acid in the final product. The acid trapped in the dried material causes it to re-dissolve when immersed in water. Photographs in Fig. 1 indicate that the chitosan materials obtained by CO_2 saturation did not dissolve in water because they did not contain acid. Only swelling of the materials can be observed, which is a well-known characteristic of this polymer. According to literature, chitosan materials can absorb liquids 30 times their own weight or more. ([42]; Zhang et al., 2019).

3.2. Antimicrobial activity

The antimicrobial activity of the obtained chitosan xerogels was determined using the modified ASTM E2149 method because of its high accuracy in relation to the given form of material [38]. Fig. 2 shows the antimicrobial properties of chitosan xerogels as a function of MW and DD against *E. coli* and *S. aureus*. The antimicrobial activity of all chitosans dissolved in lactic acid, for both Gram-positive and Gram-negative bacteria, was greater than 99.99%. The reduction of bacteria by more than five logarithmic orders proved the bactericidal activity of the materials. It can be seen that such high activity is caused by one factor, i.e. the presence of LA in the samples. The mechanism of action of this acid is based on the degradation of the cell membrane, causing leakage of proteins from the inside of the cell and, thus, cell death [43]. Stanojević-Nikolić and co-workers conducted a study to investigate the antimicrobial effect of lactic acid against different pathogen and spoilage microorganisms [powinno by]. They the determined minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against *E. coli* and *S. aureus*. For both species, the MIC and MBC were 0.25% and 0.50%, respectively [44,45]. The methodology of the antimicrobial test (number of samples, volume of applied inoculum) shows that the concentration of lactic acid in our test system was approximately 0.36%, which results in a registered killing effect (at least 3 logarithmic orders of reduction), which could also be intensified by the synergistic effect of the lactic acid and chitosan. Chitosan materials produced by the CO_2 saturation method were characterized by lower antimicrobial activity in relation to the same samples prepared using the classical method (Fig. 2), and were more effective in inhibiting the growth of the *S. aureus* than *E. coli*. Considering the MW, the antimicrobial activity against both bacterial species was similar to that obtained for materials produced using LA. MMW was characterized by the lowest antimicrobial activity, amounting to 1.35 and 0.75 logarithmic for *S. aureus* and *E. coli*, respectively, which concern materials obtained by CO_2 saturation.

Chung and Chen investigated the antibacterial activity of LMW chitosan (30 kDa) by assessing the mortality rates of *E. coli* and *S. aureus*, and demonstrated that chitosan can destroy the cell structure of both bacterial cells, resulting in the leakage of enzymes and nucleotides from

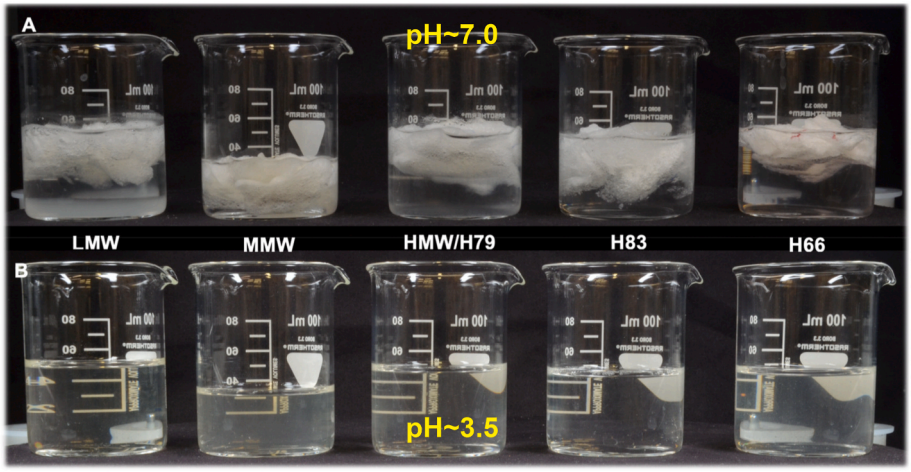

Fig. 1. Results of water extraction of xerogel materials prepared A) by CO_2 saturation, B) by dissolving with LA.

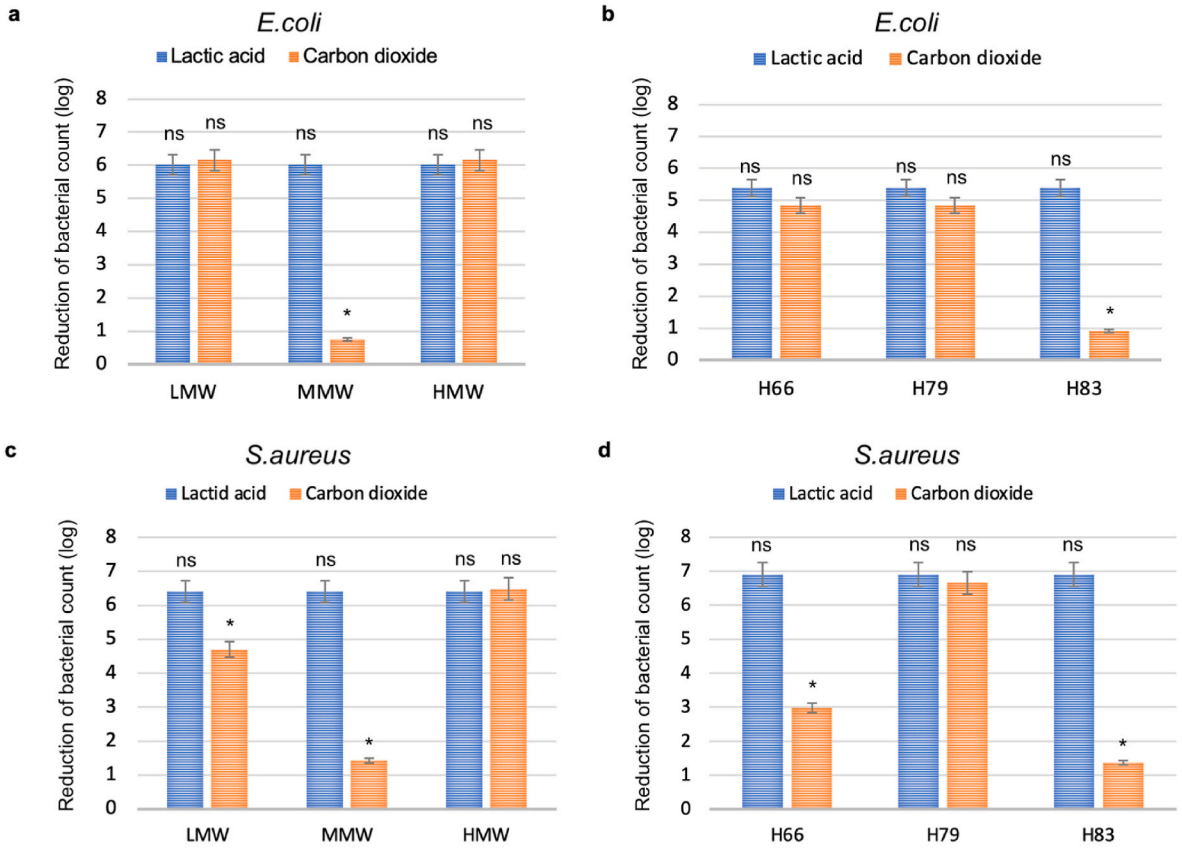

Fig. 2. Antimicrobial properties of chitosan xerogels of a) different MW against *E. coli*, b) different DD against *E. coli*, c) different MW against *S. aureus*, d) different DD against *S. aureus*. The results were analyzed by one-way ANOVA with comparisons vs. control: ns (not statistically significant, $p > 0.05$), *$p < 0.05$.

different cell locations [44]. Jeon et al. used fluorescence-labeled chitosans and monitored changes in zeta potential values of bacteria with chitosan coating, confirming the flocculation and adsorption behavior of this polymer (Jeon et al., 2001). Zheng et al. reported that the antimicrobial activity of chitosan against Gram-positive *S. aureus* increased with an increase in the MW. In addition, for Gram-negative *E. coli*, the antimicrobial activity of chitosan increased with a decrease in MW. The authors suggested the following two mechanisms for the antimicrobial activity. In the case of *S.aureus*, chitosan on the cell surface can form a polymer membrane, which inhibits nutrients from entering the cell. For *E. coli*, activity, especially in the case of low MW, is related to the penetration of chitosan into the cell [9]. If so, the antimicrobial effect must be the result of several mechanisms (Fig. 3). Moreover, differences in antimicrobial activity were also observed between chitosans H66 and H83. A bactericidal effect was achieved for both bacterial species and materials obtained using lactic acid. H83 produced by CO_2 saturation showed the lowest activity compared to H66 and H79, but it was also the chitosan with the highest viscosity and molecular weight. The H66 sample produced by the CO_2 saturation method was characterized by lower activity against S.aureus than H79 and higher than H83, which in turn could be the result of weak protonation and, at the same time, the amount of amino groups in chitosan, which determine the strength of

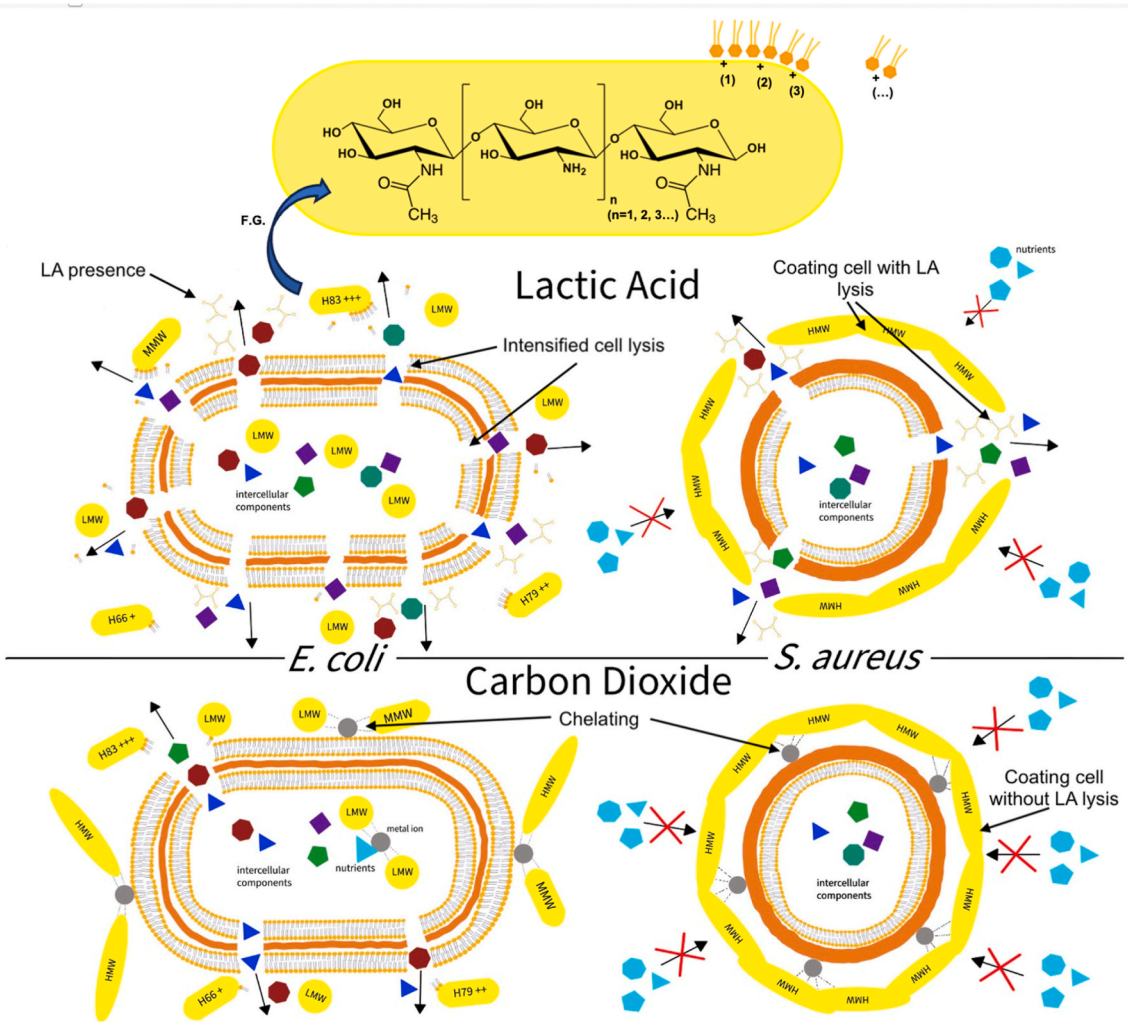

Fig. 3. Schematic showing the proposed mechanism of antimicrobial activity of chitosan depending on the method on polymer dissolution method (LA and CO_2), MW and DD against Gram-positive and Gram-negative bacteria.

the antimicrobial effect.

There is a significant difference in the structure of the cell walls of Gram-positive and Gram-negative bacteria, which leads to differences in their charge. Gram-positive bacteria have a thicker layer of peptidoglycan in their cell walls, which is surrounded by a polysaccharide and lipid layer. Peptidoglycan contains a large amount of positively charged amino acids such as lysine, which makes the cell wall of Gram-positive bacteria highly positively charged. Gram-negative bacteria are surrounded by a cell membrane composed of two lipid layers, the outer layer of which contains lipopolysaccharides (LPS). LPS is a complex molecule that consists of lipids, polysaccharides, and proteins. LPS also includes many carboxyl groups, which are responsible for the negative charge on the outer membrane of Gram-positive bacteria [46]. The positive charge of the amino group (NH3+) at pH values lower than pKa (pH < 6.3), at which this functional group carries 50% of its total electrical charge, allows interactions with the negatively charged microbial cell of the membrane, which is prone to the leakage of intracellular components [47]. In the case of chitosan prepared using CO_2 saturation (above pKa), the positive charge transfer of chitosan was negligible. This results in very limited electrostatic interactions between the polymer and cell.

Most studies have shown that an increase in DD and a decrease in pH improves the antimicrobial properties of chitosan (Jeon et al., 2001). The dependence of the increase in antimicrobial activity on the increase in DD should also be maintained for materials prepared using the innovative method of CO_2 saturation. A greater DD means a greater amount of free amino groups capable of carrying a charge, even at pH values lower than pKa (pH < 6.3).

In the case of materials prepared with LA, the change in the DD does not affect the antimicrobial activity of chitosan, which is a very high - reduction of bacteria by five and seven logarithmic orders for *E. coli* and *S. aureus*, respectively (Fig. 2b and d). There was no strict relationship between antimicrobial activity and the DD of chitosans obtained by CO_2 saturation. This may mean that DD has a lower priority in imparting activity to the materials than the MW because samples H66, H79, and H83 are high-molecular chitosans. However, the MW of H83 was significantly higher than those of the other two chitosans (H66 and H79). This may mean that molecules that are too large lose their antimicrobial activity due to the formation of aggregates (intramolecular hydrogen interactions).

For acid-free chitosans, it is highly likely that a third antimicrobial mechanism may occur, involving chelation of metal ions found on the surface of the bacteria or in its nutrients. According to the literature, this mechanism occurs more often at pH > 6.5, owing to the ability of the deprotonated amino group to donate the nitrogen lone pair. At pH values of 7–9, metal ion chelation occurs through both the amine groups and the two deprotonated hydroxyl groups, forming a more stable complex than the two $-NH_2$ receptor groups at lower pH [20].

Fig. 3 shows a diagram of the proposed mechanism of antimicrobial activity of chitosans, differentiated by the method of dissolution, MW,

DD, and type of bacteria. In the case of Gram-negative bacteria and the LA environment, the cell membrane is lysed under the influence of this acid and LMW chitosan. This causes migration of intracellular components from the cell. For Gram-positive bacteria in the same environment, there is a hybrid mechanism consisting of coating the cell with HMW, thus preventing the uptake of nutrients and damage to the cell membrane. In the case of chitosans dissolved by CO_2 saturation and Gram-positive bacteria, the cell membrane was not lysed, and only the surface of the cells was coated with HMW chitosan. For LMW chitosan and Gram-negative bacteria, membrane lysis should be limited. In addition, for all chitosans obtained by CO_2 saturation, the mechanism of chelation of ions constituting the components of the cell membranes is activated. This difference may also be due to the influence of the environment on the activity of chitosans differing in the DD, where the presence of acid enhances the interaction of polymers with the cell membrane in a series of increasing activities: H83(CO_2) < H66(CO_2) < H79(CO_2) < H66(LA); H79 (LA); H83(LA).

3.3. Cytotoxicity

The MTT assay was used to investigate the cytotoxicity of the studied chitosan xerogels against adult mouse fibroblast L929 cells at different dilutions (1:1 v/v, 1:5 v/v, and 1:9 v/v).

The obtained results were expressed as the percentage of viable cells compared to the control without the materials. The data presented in Fig. 4 clearly show that chitosan materials obtained by the LA method in a 1:1 dilution are characterized by the highest cytotoxicity against L929 cells (~20%). For the same dilution and samples prepared using the CO_2 saturation method, the viability of L929 cells was approximately 2.5 times higher (~50%). aStatistical analysis showed lower cytotoxicity of tests performed using the CO_2 method for all chitosan extracts with the lowest dilution of the extract (1:1 v/v) administered to cells. For samples with the highest dilution of chitosan extract (1:9 vv/v), no statistically significant differences were found (Fig. 4).

Hismiogullari and colleagues conducted a study to investigate the effects of organic acids, including lactic acid, on murine fibroblast cells from the NIH 3T3 cell line. Their research showed a similar relationship despite the use of different cell types. For samples administered to cells

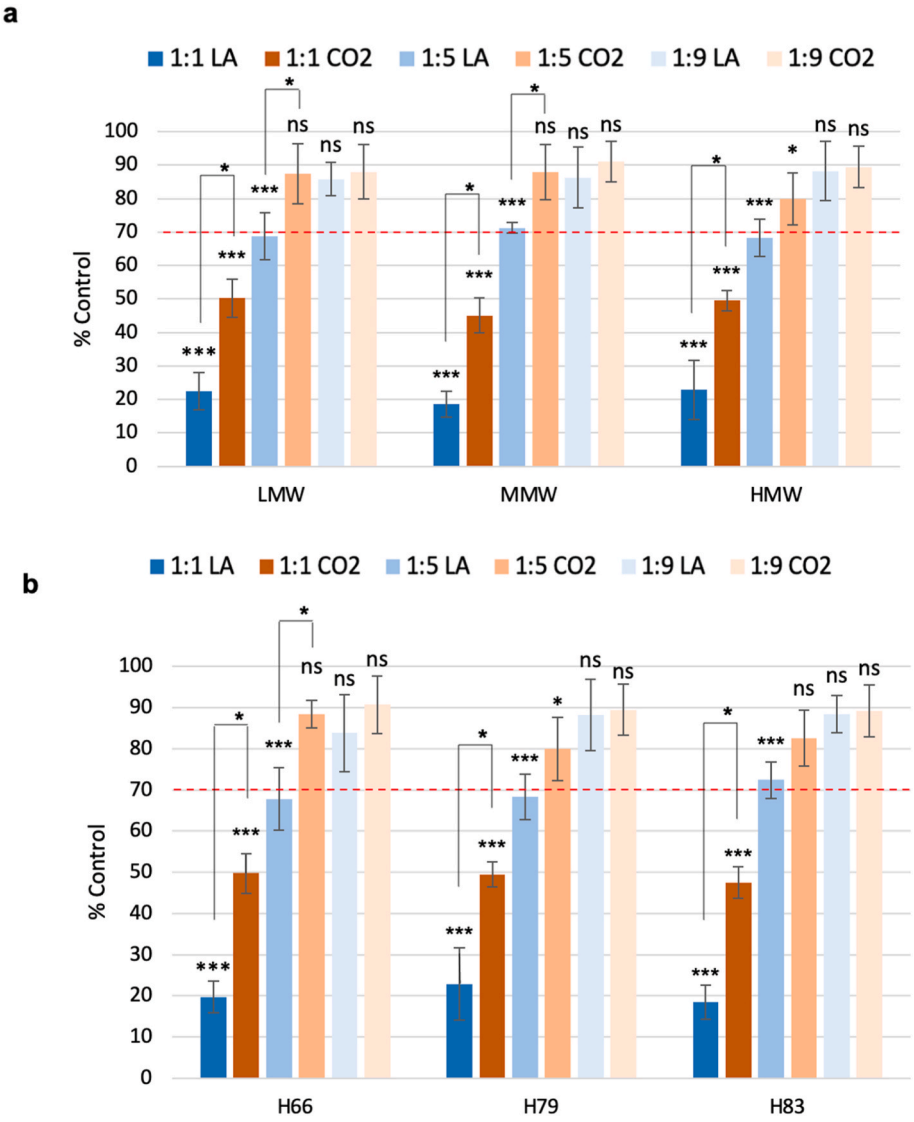

Fig. 4. The cytotoxicity of chitosan materials following 24 h incubation at different dilution factors (1:1 v/v, 1:5 v/v, and 1:9 v/v) against L929 cells. Data are expressed as the mean ± standard deviation of four independent experiments. The results were analyzed by one-way ANOVA with comparisons vs. control: ns (not statistically significant, $p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The Mann-Whitney *U* test was used to analyze the differences between the results of the cytotoxicity assay for LA and CO2—obtained materials (*$p < 0.05$).

with lactic acid concentrations of 0.5%, 0.25% and 0.1%, cell survival was ~20%, ~70% and 90–100%, respectively [48]. Comparable results were achieved for sample extracts from chitosan materials produced using lactic acid, in which the concentration of this acid was: 0.45%, 0.15%, 0.09%. Therefore, for both lines of mouse fibroblasts, the cytotoxic effect of drug acid was very similar.

Cell viability improved with increasing dilutions of extracts prepared from chitosan materials. The same trend was observed for all materials, regardless of the MW and DD of chitosan. Huang et al. also confirmed that the MW does not affect the cytotoxicity of chitosan [31]. On the other hand, cytotoxicity studies of chitosan with different MW (17 kDa, 45 kDa, and 240 kDa) by Zhang et al. showed an increase in cytotoxicity with a decrease in MW of <40%, ~60%, and >90%, respectively [49].

Results reported by Jeon et al. showed that chitosan oligosaccharides with a higher DD exhibited higher cytotoxicity against L929 cells [50]. The exact mechanism of chitosan-induced cytotoxicity in L929 cells is not fully understood and may involve multiple factors, such as cell membrane disruption through the interaction of positively charged chitosan particles with negatively charged cell membranes, induction of reactive oxygen species (ROS) generation by chitosan, activation of

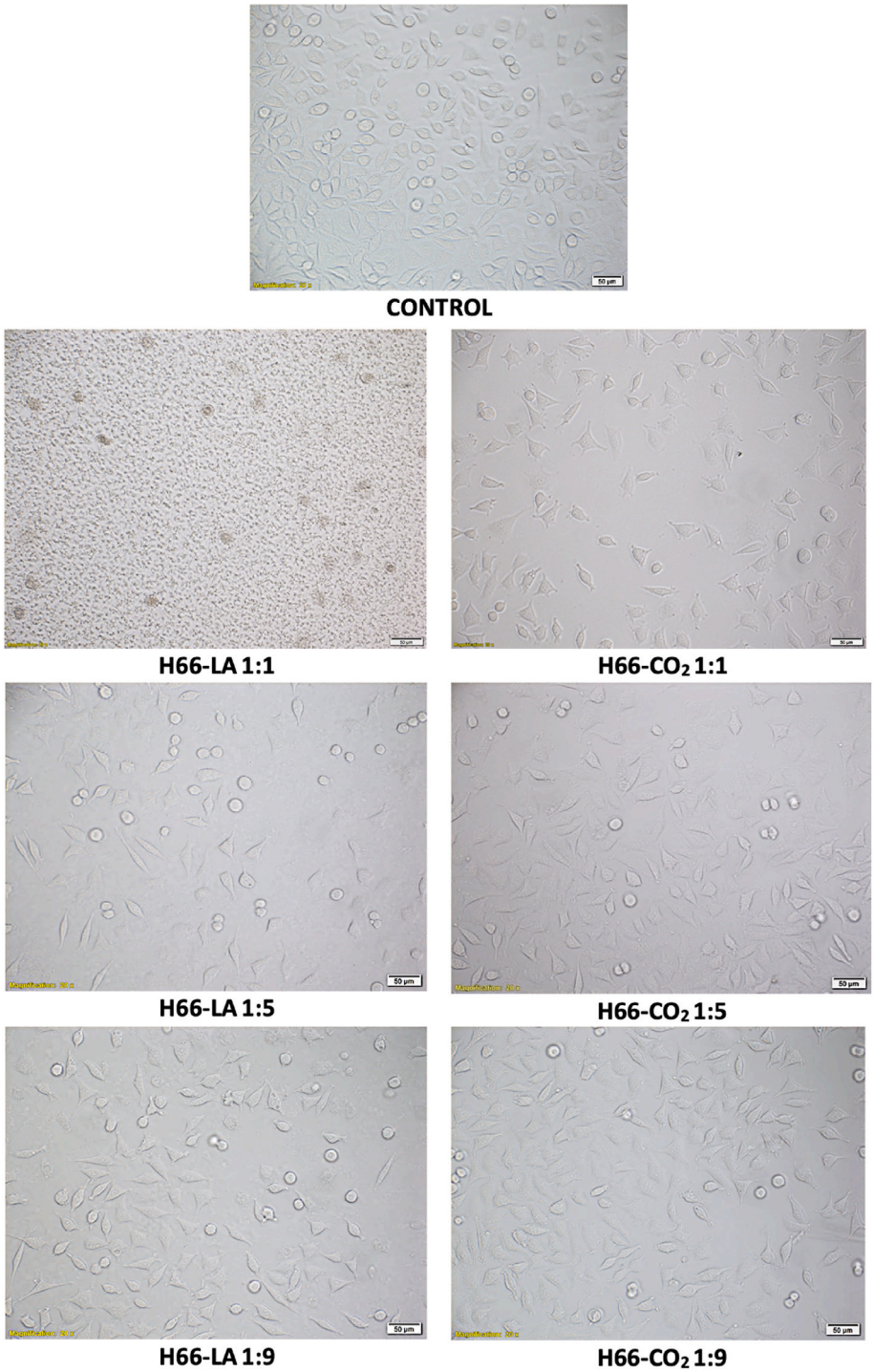

Fig. 5. Representative images at 200 × magnification of L929 cells following 24 h treatment with chitosan materials. The scale bar is 50 μm (LA-samples prepared with lactic acid, CO_2-samples prepared with CO_2 technology).

pro-apoptotic pathways or inhibition of anti-apoptotic pathways, and induction of cell cycle arrest at different stages, depending on the cell type and chitosan concentration or mitochondrial dysfunction leading to the release of pro-apoptotic factors and subsequent cell death [51,52]. The cytotoxicity data obtained here were confirmed by a morphological study of L929 cells treated with the tested chitosan extracts for 24 h (Fig. 5). Owing to the very similar cytotoxicity of all tested chitosans, the imaging results are presented in a comparison of tested dilutions only for the selected sample, H66. Fig. 5 clearly shows that as the dilution of the tested extracts increases, there is a marked increase in the number of cells visible in the microscopic images. Moreover, L929 cells showed unchanged morphology (retained their fibroblast shape) after treatment with extracts at 1:5 and 1:9 dilutions, relative to the control sample. Significant differences were observed in the morphology of cells treated with chitosan extracts at 1:1 v/v dilution. In the case of cells treated with the chitosan extract obtained by the innovative method of CO_2 saturation, the morphology of the majority of the L929 cells was preserved. The cells were oval and elongated in shape (H66–CO_2 1:1). For the H66-LA 1:1 sample, two observations were made. First, the microscopic image is blurred. Chitosan LA-extracts diluted 1:1 were characterized by the highest viscosity, resulting from the increased solubility of the polymer under such conditions, making it difficult to visualize the cell morphology. Second, the number of cells in the H66-LA 1:1 sample in the same measuring field of the microscope was lower than that in the other images, indicating substantial inhibition of cell proliferation and induction of cell death of a significant number of cells. Contours of cells with only spherical shapes are visible, revealing a change in L929 cell morphology. Undoubtedly, the cytotoxic effect of lactic acid was evident. This acid affects the function and viability of the cells, which has been confirmed in many studies. The cytotoxic effect of lactic acid is attributed to mechanisms such as the generation of free radicals, which affect the activity of intracellular enzymes, such as dehydrogenases (causing disturbances in energy metabolism), and gene expression (disruption of DNA replication and transcription) [52–54].

In vitro methods, which are crucial when evaluating medical devices such as implants, cannot capture all the complexities of the human body. Therefore, standardized tests for biosafety evaluation have been developed, as described in ISO 109933 "Biological Evaluation of Medical Devices." In the section that provides a set of recommendations, parameters and conditions for conducting the test ISO 10993–5:2009 recommends testing on a cell line derived from mouse fibroblasts L929 [55]. Accordingly, many authors in their studies focus precisely on using the L929 line as a model, a benchmark. However, in cases where authors focus their studies on a specific tissue, organ, or site of action of a compound in the body, they conduct additional studies confirming the biosafety of a given implant, on target cell lines, in order to confirm this cytotoxic effect or lack thereof.

As an example, Dodero et al. compared the effect of the method used to crosslink electrospun chitosan-based membranes on their biosafety. The toxicity of the methods was tested on three cell lines, i.e. L929, HaCaT and human Saos-2 osteoblasts. For both chemical and physical crosslinking of the test material, the survival rate of L929 cells after 24 h of contact was the highest and significantly higher than for the other cells. Moreover, only the L929 cells showed no cytotoxic effect of the samples tested. In addition, comparing the other two lines with each other, HaCaT cells had a higher survival rate after exposure to the test materials than Saos-2 cells [56]. In another study conducted by Castellano et al. the biological safety of electrospun chitosan-collagen nanofibers was also examined by MTT for two cell lines, i.e. L929 and human HaCaT keratinocytes. The study showed differential behavior of these two cell lines in interaction with different substrates over time. In the case of L929 cells, for all tested materials, a decrease in cell counts was observed after 48h, followed by an increase after 72h. Moreover, the survival rate of this cell line, compared to HaCaT cells, after 24h contact with the tested material was higher in most samples. In the case of HaCaT cells, cell abundance decreased with increasing exposure time up to 120 h [57]. The biocompatibility of the manufactured titanium alloy materials was also evaluated using the MTT test with L929 fibroblast cells and MG-63 osteoblasts. Analysis of the MTT test data showed that the survival rate of L929 cells after 120 h of incubation was significantly higher than that of MG-63 osteoblasts [58]. In contrast, bioassessment of the Ti17Mg composite material showed no difference in the abundance of viable cells of both L929 and hDPSC dental pulp stem cells, which was comparable in both cases [59]. However, in the case of cancer cells, their sensitivity is different. For example, Elsayed et al. examined the effects of Moringa oleifera seed essential oil on HeLa, human cervical cancer; HepG2, human hepatocellular carcinoma; MCF-7, human breast cancer; CACO-2, Caucasian colon adenocarcinoma and L929, mouse fibroblast cell lines. The researchers showed that cytotoxicity depended not only on the concentration of the test compound. but also on the cell line. HeLa proved to be the most sensitive cells, followed by HepG2, MCF-7, L929 and CACO-2 [60]. Similar conclusions were reached by Samarghandian, who observed that the cytotoxic effect of ethanolic saffron extract on the human non-small lung cancer cells (A549) was significantly greater than on L929 cells [61].

In conclusion, the use of the L929 line in the MTT assay to assess the biosafety of a given material is a standardized method that allows a preliminary assessment of the cytotoxic effect. One of the main reasons for using fibroblasts as a model line is that they are cells present in every tissue except blood. Therefore, if a particular compound has a detrimental effect on a fibroblast line then its use will adversely affect the entire body. In addition, these are the most commonly used cell lines in experiments which is due to their simple culture [62]. This approach makes it possible to compare specific materials, composites or compounds with each other. However, when conducting further research to evaluate the applicability of a given composite, it is necessary to conduct extended studies on their effects on specific cell lines.

4. Conclusions

Chitosan is a well-known and widely studied compound. Several of its advantages are often pointed out, such as biocompatibility, biodegradability, bioadhesiveness, coating ability, and antimicrobial activity. Due to the many mechanisms determining the resultant antimicrobial activity of chitosan and its cytotoxicity, it remains a challenge to precisely identify and link those properties.

However, it can certainly be unequivocally stated that the use of innovative technology for CO_2 saturation in the production of chitosan materials significantly expands the application possibilities of this polymer owing to the reduction of its cytotoxic effect, regardless of the MW and DD, compared to the classical method of dissolution in LA and certainly in other acid solutions. The results of the antimicrobial activity measurements indicate that the acid present in the chitosan materials, which is necessary for the dissolution and processing of this polymer, is partly responsible for its high antibacterial activity. In the next work, we plan to perform an additional test confirming the absence of the sodium salt of hydroxyacetic acid in the rinsed chitosan suspension to completely exclude the potential impact of this compound on the activity of the polymer.The results showed that the same materials produced by the CO_2 saturation technique may show much lower antimicrobial activity against microorganisms, which results from the resultant mechanisms related to the MW of the polymer and the DD. Nevertheless, the antimicrobial effect of materials produced by CO_2 saturation is still sufficient to design bacteriostatic or even bactericidal materials.

The results indicate that chitosan is a safe raw material and can be used in many industries, even in the food industry (registered as a food additive in some countries), as well as in the packaging industry. However, it is an organic compound of natural origin, which is often difficult to process technologically and ensure appropriate mechanical properties of the final products. This creates the need to use it as an additive and not the main raw material, which still requires strict

control, including potential cytotoxic effects.

Author contributions

Conceptualization: S.M., Data curation: S.M., A.B–K., K.S., J.K., E.A., and R.T., Formal analysis: S.M., J.K., Funding acquisition: S.M., Investigation: S.M., A.B–K., K.S., J.K., Methodology: S.M., A.B–K., K.S., J.K., E.A., and R.T., Project administration: S.M., Resources: S.M., A.B–K., K.S., J.K., E.A., and R.T., Software: S.M., A.B–K., K.S., J.K., Supervision: S.M., E.A. and R.T., Validation: S.M., A.B–K., K.S., J.K., E.A., and R.T., Visualization S.M., K.S., J.K., Writing—original draft preparation: S.M., A.B–K., K.S., J.K., E.A., and R.T, Writing—review and editing: S.M., A.B–K., K.S., J.K., E.A., and R.T All authors have read and agreed to the published version of the manuscript.

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.

Data availability

Data will be made available on request.

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