SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the interaction of chitosan applied in microcapsules with probiotic bacteria

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Content

1. Introduction

The importance of probiotic bacteria has increased recently and is still a research area that uses significant resources. Probiotics are living microorganisms that, when used in the right amount, have a beneficial effect on the body. The use of these probiotic microorganisms is also of great importance in animal husbandry or human medicine. In animal husbandry, its main role is to mix it with feed, to achieve an increase in yield or to improve and maintain the general health of the herd. It was a significant step forward that antibiotics that had been used so often until then, such as tetracycline antibiotics, were omitted from the feed. These antibiotics were often used not to treat infections, but to increase faster growth and muscle mass. However, such a procedure carries a significant risk. The development of antibiotic resistance can be a negative result of the use of subtherapeutic doses.

Probiotic microorganisms used to induce unwarranted antibiotic use are much less dangerous. Essentially, it can be considered a supportive procedure, where the bacterial environment of the body is influenced in such a way as to reduce the living space of pathogens with beneficial bacteria, as well as to reduce the host's susceptibility to diseases through immunomodulatory effects.

When choosing a probiotic microorganism, a very important question is how it can survive and reproduce after being exposed to the often not very favourable conditions inside the body. Even though it is such an important question, to what extent the viability of the feed or medicine is reduced during formulation, whether the micro-organism remains in the right amount to be able to exert its beneficial effect on the body.

The human-animal ecosystem is in constant interaction with each other through its microbiomes. The European One Health Joint Program (OHEJP) is a scientific collaborative research program co-funded by the European Commission that aims to help prevent and control human health through joint action against foodborne zoonoses, antimicrobial resistance, and emerging microbiological threats influencing, food-borne and environmental contaminants. Recognizing, evaluating, and treating health hazards and risks at the human-animal-ecosystem interface is only possible on a global basis, with the cooperation of several specialist fields.

2. Aims

The aim of my PhD research work:

1. Investigation of the viability of probiotic bacteria strains (*E. faecium*, *B. bifidum*, *L. plantarum*) and its increase, under the heat effects occurring during the formulation of animal feeds and the conditions appearing in the gastrointestinal tract *in vivo*. Alginate-based microcapsule formulation that functions as a carrier system for probiotic strains. We planned to improve the acid and heat resistance of the probiotic microcapsules by coating them with chitosan. We want to investigate the viability and thermotolerance of cells under dry and wet heat treatment.

Due to the unexpected results obtained in the initial phase of the research work, our further tests will be carried out exclusively with microcapsules containing *L. plantarum*.

2. In order to reveal unexpected results, the microcapsules containing *L. plantarum* are not only coated with chitosan but another group of microcapsules is coated with Eudragit L100-55. In order to examine the effectiveness and intact nature of the coating, we planned to characterize the uncoated and coated microcapsules with a light and fluorescence microscope and to examine the surface characteristics with a Scanning Electron Microscope (SEM). Carrying out elemental analysis to determine the quality of the coating formed on the surface of the microcapsules.

3. The dissolution tests were performed in artificial gastric and intestinal juices with different microcapsule formulations in their coatings. We plan to examine the samples of the dissolution experiment using two methods. After quenching the sample, the viability is determined by CFU determination, and the ratio of live/dead cells and the release profile of the cells are determined by flow cytometry.

4. The low molecular weight chitosan used in our tests is labeled with FITC to determine the amount of dissolved chitosan.

5. Our further tests are carried out with three chitosans of different molecular weights (LMW, MMW, HMW) to examine the presumed interaction between bacterial cells and chitosan-based on the results so far. We determine the MIC value for *L. plantarum* with all three chitosans and then examine the killing kinetics and the ratio of live/dead cells using flow cytometry with the same samples.

6. We consider it important to learn about the interaction of chitosan with *L. plantarum* cells to understand the mechanism of the antibacterial effect, which is examined as a function of chitosan concentration and molecular weight. We examine membrane integrity and zeta potential changes to analyze this mechanism. The interaction of chitosan with the cell wall of *L. plantarum* is visualized using confocal microscopy using FITC-labeled chitosan.

3. Materials and methods

Three species of probiotic bacteria were applied, such as *L. plantarum subsp. plantarum* (ATCC 14917) from ATCC (American Type Culture Collection) (Manassas, VA, USA), *B. bifidum*, and *E. faecium* provided by Dr. Judit Remenyik (Institute of Food Technology, University of Debrecen, Debrecen) provided for us. Low molecular weight chitosan (50–190 kDa; 20–300 cp; LMW), medium molecular weight chitosan (200–800 cp; MMW), high molecular weight chitosan (310–375 kDa; 800–2000 cp; HMW) were from Sigma-Aldrich obtained from Sigma-Aldrich (Budapest, Hungary)

Using three strains of probiotic bacteria (*L. plantarum*, *B. bifidum*, and *E. faecium*), we prepared single-component alginate microcapsules, which were coated with LMW chitosan and lyophilized. After lyophilization, the other part of the microcapsules was coated with gastric juice-resistant Eudragit L-100-55. The microcapsules were produced and coated at the Department of Pharmaceutical Technology. The fluidization procedure was carried out with the help of Dr. Ádám Haimhoffer and Dr. Bernadett Gál.

The morphology of the wet microcapsules was examined with a light microscope and a fluorescence microscope. The size and morphology of the dry microcapsules were characterized using a scanning electron microscope (SEM). The SEM images and the elemental analysis were made at the Technical Faculty of the University of Debrecen, with the help of István Budai.

The thermotolerance of the probiotic bacteria was tested with dry heat at 80 °C for 10 minutes and at 100 °C for 5 minutes in a peptone water medium at 80 °C for 1 minute and dissolved in peptone water, then their CFU value was determined.

The dissolution test of the microcapsules was performed in simulated gastrointestinal fluid. In this experiment, we only worked with microcapsules containing *L. plantarum*. The microcapsules were divided into three groups: uncoated alginate; coated with chitosan and coated with Eudragit L100-55. The microcapsules were filled into Torpac® gelatin capsules, and the microcapsule groups were further divided into two parts, Eudragit L100-55 coated and uncoated gelatin capsules. The dissolution test was performed in artificial gastric (pH 2.0) and intestinal fluids (pH 7.4). The pH 2.0 medium was removed after 60 minutes and replaced with artificial intestinal fluid, in which the dissolution was also carried out for 60 minutes. Dr. Bernadett Gál and Dr. Gábor Vasvári helped me fill and coat the gelatin capsules.

The release of *L. plantarum* from the microcapsule was investigated in simulated gastric (pH 2.0) and intestinal juice (pH 7.4). The pH of the acidic solution was adjusted to 7.4 with sodium hydroxide after 60 minutes of exposure. Additional samples were taken at 120, 180 and 240 minutes of the total release time. The samples were centrifuged and released bacterial cells were stained with SYTOX Green reagent and examined with a flow cytometer. The number of released cells and the ratio of live/dead cells were evaluated using GraphPad Prism 5.0 software. The flow cytometric measurements were performed at the Department of Pharmaceutical Technology, with the help of Dr. Ferenc Fenyvesi.

For labelling of chitosan with FITC, chitosan was dissolved in 0.1 M acetic acid solution, FITC in methanol. FITC solution was added to the acidic aqueous solution. The reaction mixture was kept in the dark for 3 hours, and the FITC-labelled chitosan was precipitated with 1 M NaOH. The precipitate was filtered and dialyzed with deionized water. The dialysis-purified product was then freeze-dried. The labelling efficiency was determined with fluorimeter. The FITC labelling of chitosan was carried out by Dr. Ilona Bakai Bereczki at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Debrecen. The purification process and determination of labelling efficiency were carried out at the Department of Pharmaceutical Technology. FITC-labelled chitosan was used to quantify the chitosan coating of the microcapsule and for confocal microscopy studies.

The membrane integrity of *L. plantarum* was investigated by measuring the released substances at 260 nm. Chitosan solutions of different concentrations and molecular weights were added to the bacterial suspensions in a 1:1 volume ratio. The release of metabolic substances was measured on a microplate reader at 260 nm every 10 minutes for 3 hours. With the same sample preparation, zeta potential determination was also performed on a Malvern Nano-ZS Zetasizer, however, the samples were washed and centrifuged to remove excess chitosan.

Chitosan solutions of different concentrations were mixed with bacterial suspension in a volume ratio of 1:1 and the samples were incubated at 37 °C for 60 minutes. 25% of the chitosan concentration was measured from FITC-labeled chitosan, the excess FITC-chitosan was removed by centrifugation. The bacterial cell membrane was stained with FM®4-64 dye. We were assisted by Dr. György Vámosi at the Institute of Biophysics and Cell Biology of DE ÁOK in the preparation of the confocal microscopic images.

4. Results

4.1. Thermotolerance test with dry and wet heat treatment

The heat tolerance of different probiotic bacteria was investigated under three different heat exposure protocols. In all three conditions, it can be established that *L. plantarum* and *B. bifidum* showed a similar sensitivity, while *E. faecium* showed a different sensitivity pattern in the case of uncoated and chitosan-coated microcapsules.

The survival rates of *L. plantarum* and *B. bifidum* in uncoated microcapsules under wet heat stress in peptone water were 13.33 ± 0.023% and 16.66 ± 0.59%, while in chitosan-coated microcapsules 0.33 ± 0.045% and 0. It was 03 ± 0.008%. The survival rate of *E. faecium* in uncoated microcapsules was 0.36 ± 0.042%, while that in chitosan-coated microcapsules was 5.36 ± 0.87% after wet heat exposure.

4.2. Effect of storage on the viability

The change in CFU values over time was shown on a logarithmic curve. Time t_0 was taken as the day of preparation of the wet microcapsules, during which the *L. plantarum* cells were mixed into the alginate solution. After lyophilization of the microcapsules, the samples were kept at 4 °C. The viability of probiotic bacteria changes during storage, so we recorded a storage time – CFU curve from the uncoated alginate group, on which we followed the change in cell viability as a function of storage time. The "raw" CFU values obtained in each of our experiments were converted into percentage values considering the storage time of the sample.

4.3. Characterization of coated and uncoated microcapsules

The shape and size of the wet alginate microcapsules were analyzed with a light microscope. The average value of wet alginate microcapsule diameter was 412.54 ± 28.83 µm using a 150 µm nozzle. The shape of wet microcapsules is isometric, almost spherical, surrounded by a shell.

After freeze-drying the microcapsules, the size of the solid particles was determined by SEM. The average diameter of uncoated alginate microcapsules was 187.21 ± 24.36 µm, chitosan-coated 203.5 ± 31.98 µm, and Eudragit-coated 312.6 ± 50.36 µm (n = 50.36 µm). The surface of the dried microcapsules is slightly rough, ribbed, which makes the surface irregular, its shape is almost isometric, and in some places, it is slightly elongated due to the twins formed

during drying. The surface of the microcapsules coated with chitosan was slightly ridged, while the surface of the samples coated with Eudragit L100-55 was almost smooth.

4.4. Elemental Spectroscopy

The effectiveness of the coating and the surface of the microcapsules were examined by photoelectron spectroscopy. By analyzing the spectrum of the surface of the uncoated microcapsule, we can confirm the presence of calcium. To develop microcapsules, we used sodium alginate, which was gelled in calcium chloride. However, the spectrum showed no Na signal, which indicates the proper precipitation and crosslinking of alginate. After chitosan coating, the signal intensity of calcium and chlorine decreased significantly, which indicates an effective chitosan coverage. X-ray analysis confirmed that the main peaks in the chitosan spectrum are C and O, which are the main element content of chitosan. The calcium signal of the surface of the microcapsule coated with Eudragit disappeared, but the signals of C and O became stronger. Based on elemental analyzes of the surface, we can assume the appropriate and effective coating in the case of chitosan and Eudragit as well.

4.5. Dissolution tests

We investigated the effect of the pH of the dissolution medium on the different coatings, as well as the effect of the coating on the % survival rate of *L. plantarum*. The dissolution was carried out for 60 minutes in each dissolution medium. During the dissolution test in pH 2.0 medium, the surface of the Eudragit L100-55 coated gelatin capsules did not swell and no macroscopic signs of dissolution were visible. However, the uncoated gelatin capsules began to swell in acidic media. After 60 minutes, the pH 2.0 release medium was changed to a pH 7.4 medium, at which time the dissolution of the enteric-coated gelatin capsules began and swelling of the gelatin was observed. There was no significant difference (89.81 ± 5.64) in the survival of alginate microcapsules filled in gelatin capsules coated with Eudragit L100-55 compared to the control. Similarly, no difference in survival was observed for microcapsules coated with Eudragit L100-55 compared to controls (95.09 ± 4.73) or for gelatin capsules coated with Eudragit L100-55 (99.09 ± 9, 27). The viability of *L. plantarum* in chitosan-coated samples was completely inhibited, so the survival rates in chitosan-coated and uncoated gelatin capsules were practically zero.

4.6. Determination of Dissolved Chitosan Coat

Considering the labeling efficiency and the proportion of labeled chitosan in the coating solution, the concentration of chitosan in the solvent was 0.043%, which is 431 ppm. The

calculated value was determined after dissolving the microcapsules in a pH 2.0 medium for 60 minutes. The chitosan coating was dissolved in an acidic medium, then the pH of the dissolution medium was changed to 7.4, and a sample was taken at 240 minutes. The degree of deacetylation of chitosan is 75-85%, and its molecular weight is 50-190 kDa. We were the first to determine the concentration of the chitosan coating released from the surface of the microcapsules, we did not have previously published literature data on this.

4.7. Determination of cell release profiles and live/dead cells

In this experiment, the release of bacteria was investigated as a function of pH and coating. Samples kept in a pH 2.0 release medium showed low cell counts after 60 min with no significant difference. When the pH was modified to 7.40, the release was measured in the uncoated microcapsules, 42.86% of the initial bacterial concentration was released in 240 minutes. In the case of the chitosan-coated samples, a similar release kinetics was observed in the initial phase as in the uncoated microcapsule samples, but at 240 minutes a higher release (71.43%) was measured than in the Eudragit L100-55 coated samples. In the case of Eudragit L100- 55– coated samples, the fastest release was measured after adjusting the pH of the release medium to 7.40, so 80% of the *Lactobacillus* were released. The release of bacterial cells after the pH changes after 60 minutes showed a significantly different mechanism. The dissolution of the chitosan coating in a pH 2.0 medium did not result in a significantly different dissolution profile compared to the uncoated alginate microcapsules, except for the last measurement point (240 min). The enterosolvent coating from the surface of the Eudragit L100-55 coated microcapsules started to dissolve only after the pH change, which resulted in an explosive release of bacteria at 120 minutes followed by a slower saturation phase. After sampling (60, 120, 180, and 240 minutes), the bacterial cells were stained with SYTOX Green, and the ratio of live/dead cells was examined. For uncoated alginate and Eudragit L100-55 coated microcapsules, no significant difference in viability was observed at the sampling times. However, after 60 minutes, the bacteria showed higher mortality in the samples coated with chitosan, which remained characteristic throughout the study. To determine viability, the sum of live and dead cells, i.e. the number of released bacteria, was considered 100% for each sample.

4.8. MIC value determination and Time-kill Assay

The MIC values of the tested chitosan compounds were 0.007% for low, medium, and high molecular weight chitosan. All examined chitosan of different molecular weights exerted a

pronounced inhibitory effect on *L. plantarum* cells in a concentration-dependent manner. A remarkable bactericidal effect (at least a three-log reduction in the number of live cells compared to the initial inoculum) was observed from 0.015% chitosan at all molecular weights.

Further tests were carried out with three chitosans of different molecular weights. The viability of the bacterial cells was also determined using a flow cytometer in the present study based on the ratio of live/dead cells. In the case of the control and the samples with the lowest (0.001%) chitosan concentration, no significant difference was observed in the viability of *L. plantarum*. This can be established by examining the chitosan of all three molecular weights. However, with a tenfold increase in the chitosan concentration (0.01% and 0.1%), we noticed a significant decrease in viability.

Regarding the molecular weight of chitosan, the viability showed a decreasing trend with increasing molecular weight. The ratio of live/dead cells stabilized at 8 hours in the case of low molecular weight chitosan and at 12 hours in medium molecular weight chitosan. In the case of high molecular weight chitosan, stabilization was observed in the 24-hour result.

4.9. Membrane integrity test

The destabilizing effect of chitosan on the membrane was investigated in the *L. plantarum* membrane integrity test, and the amount of released nucleic acid was measured at 260 nm. In the case of the untreated bacterial cells, a small, linear increase in absorbance was observed. A lower absorbance was observed for all three chitosan molecular weights at a concentration of 0.001% compared to the control. During the treatment with a concentration of 0.1%, we found a maximum absorbance of the samples at 30-40 minutes, and then we measured a decreasing trend. These characteristics are more pronounced in the case of low and high molecular weight samples, while less so in the case of medium ones. The absorbance of the samples at a concentration of 0.01% changed similarly to the untreated control.

4.10. Determination of zeta potential on the surface of the bacterial cell

The zeta potential was determined on the surface of *L. plantarum*. In the samples containing untreated *L. plantarum*, the value of the zeta potential was -15.15 ± 0.49 mV, which became more positive after chitosan treatment. The zeta potential showed a concentration dependence for all three molecular weight chitosans. The largest zeta potential change was obtained with the 0.1% chitosan treatment (LMW: -4.10 mV, MMW: -3.52 mV, HMW: -2.96 mV). Comparing the results obtained with chitosan of different molecular weights, we did not find any significant difference in the zeta potential values.

4.11. Investigation of chitosan-cell membrane interaction with confocal microscopy

The *Lactobacillus* cells were treated with the concentration of chitosan used in the previous studies (0.001-0.1%), and treatment with a higher concentration (1%) was also used. Chitosan was labeled with FITC for detection. In the case of the lowest chitosan concentration, an inhomogeneous distribution was observed on the bacterial cells, near the cell wall, while FM ® 4–64 used for membrane staining also showed an uneven distribution, which suggests membrane aggregation. At the lowest concentration, we observed not only membrane aggregation but also the changed shape of the cells, indicating cell damage. Chitosan in a higher concentration (0.01-1%) is evenly distributed on the surface of the bacterial cell wall, rather than forming a uniform coating. In addition to the uniform staining of the membrane, we observed the normal morphology of the cells, without membrane damage.

5. Discussion

The aim of our study was to create a carrier system for probiotic bacteria that can be used in feeding, which increases their thermostability and acid resistance The increase in thermostability is necessary due to the heat load of around 80-100 °C occurring during feed extrusion, while the increase in acid resistance is because of the low pH in the stomach reducing viability. As a solution, we chose to combine the bacteria embedded in the alginate-based microcapsule and the gastric acid-resistant coating applied to the microcapsule.

We started our tests with microencapsulation of three probiotic strains. These microcapsules were coated with chitosan and the thermal load of the samples was examined. We could observe the same tendency when examining the different thermal load procedures. However, we observed a significant difference in the response of different probiotic strains to chitosan coating. While the viability of *L. plantarum* and *B. bifidum* decreased, the viability of *E. faecium* showed an increase. These unexpected results with the chitosan coating led our work in a new direction. *L. plantarum* and *B. bifidum* showed similar sensitivity tendencies, therefore in the rest of our work, we only performed tests using microcapsules containing *L. plantarum* to explore the processes underlying the reduction in viability caused by coating. The dissolution tests were not performed in the pH 2 hydrochloric acid solution recommended in Ph.Hg.VIII., but in PBS adjusted to pH 2, as this is closer to physiological conditions. Before the dissolution tests, the success of the application of both the chitosan and the Eudragit L100-55 coating was examined with SEM images, which was also confirmed by the results of the elemental analysis. After the successful coating, we examined the effectiveness of the different coatings. Again, a surprising result was obtained in the case of microcapsules coated with chitosan. At the end of the dissolution test, there were not enough viable *Lactobacillus* in the samples that would have been able to form colonies, so the CFU value was practically zero.

Further studies were performed, in which the release of *L. plantarum* from the microcapsule and the ratio of live/dead cells were examined by flow cytometry. These results were correlated with CFU results, which were determined from samples taken from the same experiment. In the case of samples coated with chitosan, we experienced a slow release of bacterial cells, which was presumably caused by chitosan gelling, preventing the diffusion of bacteria. In contrast, in the case of microcapsules formulated with Eudragit L100-55 coating, sodium lauryl sulfate, and polysorbate in the coating could help the dissolution of alginate but did not reduce the viability

of the bacteria. The concentration of microcapsules in the dissolution medium is a relevant factor because it determines the concentration of chitosan appearing in the dissolution medium. Therefore, we used the same concentration of microcapsules/dissolution medium for both the CFU determination and the cytometry. The concentration of chitosan in a dissolution medium has not been investigated so far, nor has it been compared with the MIC value of chitosan. The MIC values of the probiotic strains were less investigated, we found one publication that gave the MIC values for three strains of *Lactobacillus* for chitosans of different molecular weights. H.K. No et al. for 28–224 kDa chitosan: MIC values of 0.05–0.05% for *L. plantarum*, >0.1–0.08% for *L. brevis,* and 0.1–0.1% for *L. bulgaricus* were given. We determined the MIC values of different molecular weights of chitosan. According to our calculations, the chitosan released from the microcapsule resulted in a concentration of 0.043% in the solvent. For the preparation and our experiments, we used chitosan with a mass of 50-190 kDa, which is the same order of magnitude as that given by H.K. No et al. (28–224 kDa). Based on the compared data, it can be assumed that the concentration of dissolved chitosan in the release medium exceeds the MIC value of *L. plantarum*. In addition to the amount of chitosan coating, several other factors affect the number of viable bacteria, such as the duration of the dissolution test and the duration of lyophilization. In the thermotolerance test, the viability of *E. faecium* in chitosan-coated samples was better than in uncoated alginate microcapsules. This may be due to its higher MIC value for chitosan.

We wanted to verify our hypothesis with further studies, which were no longer conducted with a microcapsule formulation but focused on the interaction between dissolved chitosan and *L. plantarum*. We wanted to investigate the mechanism of the antibacterial effect experienced in the case of *L. plantarum*. In this series of tests, we first determined the MIC values of three chitosan products with different molecular weights (LMW, MMW, and HMW) on *L. plantarum*. In the microdilution experiments, the MIC values for all three chitosans were 0.007%. Thus, our previous assumption that chitosan appears in a concentration above the MIC value in the release medium during the release tests was confirmed. By analyzing the time-kill curves, no bactericidal effect was established at the lowest concentration (0.003%), which correlates with the MIC result. The viability of *L. plantarum* was not reduced at the lowest concentration treatment (0.003 m/v%), but at higher concentrations (0.015-0.125 m/v%) chitosan reduced it to 25-75%.

Studies of chitosan's membrane-damaging effect were consistent with membrane permeability and TEM microscopic studies performed by others. The interaction of *L.*

plantarum with 0.1%, 0.01%, and 0.001% chitosan solutions were also investigated. When using the 0.001% chitosan solution, a low absorbance was measured, while at the medium concentration (0.01%) it was similar to the control, but these curves do not show a clear nucleic acid release as in the case of the highest concentration. This suggests that the bacterial cell wall becomes permeable only after the 0.1% treatment, which can probably be attributed to the bactericidal effect based on the time-killing results above. No nucleic acid release from the cytoplasm was observed in the lower concentration treatments, which indicates a bacteriostatic interaction in the 0.01% treatments. In our studies, we did not observe a significant difference in nucleic acid release between chitosan products of different molecular weights; however, the maximum on the curve of medium molecular weight chitosan is elongated, and nucleic acid release is lower compared to low and high molecular weight chitosan. This is probably because medium molecular weight chitosan is a physical mixture of low molecular weight and high molecular weight chitosan, giving a medium viscosity product.

In the case of *L. plantarum*, as a Gram-positive bacterium, we aimed to obtain more detailed information on the mechanism of interaction and inhibition. Due to the polycationic nature of chitosan, it is expected that negatively charged bacterial cell walls will interact with chitosan. By measuring the zeta potential, we examined to what extent this interaction affects the charge of the surface of the bacterial cell wall. Based on the results, it can be concluded that the chitosan remained on the surface of the bacterial cells even after washing the samples, as the surface of the bacterial cells became more and more positive as the concentration of chitosan increased. This suggests that the higher the concentration of chitosan, the better it covers the surface of the bacteria. At higher concentrations, due to the entanglement of the polymer chains, chitosan here behaves as if it were a long chain with a high molecular weight. Due to its large size, it cannot enter the cell, but interacts with it extracellularly. Probably, this long-lasting interaction results in an increase in the permeability of the bacterial cell membrane.

The nature of the interaction was also investigated using a confocal microscope. The images clearly show that at a lower concentration (0.001%) chitosan is not evenly distributed on the surface of the bacterial cell. In addition, we observed similar aggregates in the bacterial membrane overlapping with FITC-chitosan aggregates. The membrane structure, which is thickened in some places, may also indicate internalization at a low concentration because of the chitosan entering the bacterial cell. However, at a concentration of 0.001%, chitosan probably does not cause a bactericidal or bacteriostatic effect either, since we did not observe a significant decrease in CFU values at a concentration lower than the MIC. Nevertheless, in the

low molecular weight chitosan samples, the CFU is slightly lower at the beginning of the test, which may indicate some inhibitory effect.

6. Summary

Microencapsulation of probiotic bacteria has many advantages. It can improve viability, facilitate storage and administration, and can be released in the gastrointestinal system at the right place and time in a targeted manner. However, during examination and formulation, some factors must be considered, as they can significantly influence the test results and the effectiveness of the preparation.

In my doctoral work, I investigated the viability-reducing effect of chitosan applied as a microcapsule coating agent on probiotic strains, mainly *Lactobacillus plantarum*. Based on my work, I made the following conclusions:

1. The chitosan coating dissolved from the surface of the microcapsule and reduced the viability of *Bifidobacterium bifidum* and *Lactobacillus plantarum*. Due to this viability-reducing effect, the assessment of the thermoprotective role of chitosan for these two strains is not established. In the case of *Enterococcus faecium*, I verified the thermostabilizing effect of chitosan.
2. During the dissolution of microcapsules, containing *L. plantarum* coated with chitosan, in artificial gastric and intestinal juice, I experienced a significant decrease in viability.
3. I determined the amount of chitosan coating released from the surface of the microcapsule using a FITC–labeled chitosan for the first time. I found that the concentration of chitosan in the release medium is at least five times higher than the MIC value of *L. plantarum.*
4. I verified that the viability-reducing effect of chitosan on *L. plantarum* is concentration-dependent. However, I found no significant differences among the viability-reducing effect of the chitosan derivatives with different molecular weights that I used.
5. I proved that chitosan establishes an irreversible connection with the membrane of *L. plantarum* cells, which interaction results from the polycationic nature of chitosan. The viability-reducing effect of chitosan at a concentration of 0.1% is related to the reduction of membrane integrity. I confirmed the adsorption of chitosan to the cell wall of *L. plantarum* for the first time with confocal microscopy.

Based on my results, I recommend that the determination of the concentration and MIC values of the excipients used in the preformulation tests of the probiotic preparations is necessary. Therefore, we can predict the expected effectiveness and stability of the preparation.

7. List of publications

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Registry number: DEENK/121/2023.PL
Subject: PhD Publication List

Candidate: Lóránd József Erdélyi
Doctoral School: Doctoral School of Pharmacy
MTMT ID: 10083516

List of publications related to the dissertation

1. Kovács, R. L.*, **Erdélyi, L. J.***, Fenyvesi, F., Balla, N., Kovács, F., Vámosi, G., Klusóczki, Á., Gyöngyösi, A., Bácskay, I., Vecsernyés, M., Váradi, J.: Concentration-Dependent Antibacterial Activity of Chitosan on Lactobacillus plantarum.
Pharmaceutics. 15 (1), 1-11, 2022.
DOI: http://dx.doi.org/10.3390/pharmaceutics15010018
* These authors contributed equally to this work.
IF: 6.525 (2021)

2. **Erdélyi, L. J.**, Fenyvesi, F., Gál, B., Haimhoffer, Á., Vasvári, G., Budai, I., Gálné Remenyik, J., Bereczki, I., Fehér, P., Ujhelyi, Z., Bácskay, I., Vecsernyés, M., Kovács, R. L., Váradi, J.: Investigation of the Role and Effectiveness of Chitosan Coating on Probiotic Microcapsules.
Polymers. 14 (9), 1-16, 2022.
DOI: http://dx.doi.org/10.3390/polym14091664
IF: 4.967 (2021)

List of other publications

3. **Erdélyi, L. J.**, Váradi, J.: Lokális antibiotikum-felhasználás csökkentése, illetve kiváltása sebkezelésben.
Gyógyszerészet. 67, 199-202, 2023.

Total IF of journals (all publications): 11,492
Total IF of journals (publications related to the dissertation): 11,492

DEBRECENI EGYETEM Egyetemi és Nemzeti Könyvtár

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

24 April, 2023

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