Development of Chitosan Nanoparticles with Antimicrobial Activity Against *Listeria monocytogenes*

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Universidade do Minho

Escola de Engenharia

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Dissertação de Mestrado
Mestrado em Tecnologia e Ciência Alimentar

Trabalho efetuado sob a orientação de:

Doutor Alejandro Garrido-Maestu

Doutora Maria do Pilar Teixeira

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Direitos de Autor e Condições de Utilização do Trabalho por Terceiros

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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Resumo: Desenvolvimento de nanopartículas de quitosano com atividade antimicrobiana contra *Listeria monocytogenes.*

A *Listeria monocytogenes* é um dos patogénicos alimentares mais perigosos devido à sua resistência a condições ambientais adversas e, com base na gravidade, a listeriose foi a mais grave, com a maior taxa de hospitalização e mortalidade, entre as quatro doenças zoonóticas mais comuns relatadas na União Europeia em 2019. Os antibióticos têm sido essenciais para sustentar a saúde humana e animal. A OMS considerou que o nível de resistência aos antibióticos na última década é uma séria ameaça à saúde global.

Assim, o uso de compostos naturais com atividade antimicrobiana é de interesse, por isso, neste trabalho focou-se a atenção no quitosano, pois é aprovado pela FDA como Geralmente Reconhecido Com Seguro (GRAS) e possui propriedades antimicrobianas. As nanopartículas de quitosano (CN) têm uma maior disponibilidade de interação com outras moléculas e células bacterianas, além de serem capazes de reagir em pH ácido ou básico. A atividade antibacteriana das CN é inespecífica e, portanto, essas nanopartículas foram funcionalizadas com anticorpos monoclonais (mAb) para melhorar o seu direcionamento ativo com o patogénico alvo, ou seja, *L. monocytogenes.* Neste projeto, foi utilizado o mAb C11E9, que se liga fortemente à parede celular bacteriana de *L. monocytogenes*.

Este projeto visou avaliar as propriedades antimicrobianas de nanopartículas de quitosano sintetizadas a partir de diferentes origens e tentar melhorar as suas propriedades contra *L. monocytogenes*, funcionalizando-as com moléculas bioativas.

Para a fabricação de nanopartículas foi utilizado o Protocolo de Gelificação Iónica, e foram selecionados três quitosanos de baixo peso molecular, Sigma, HMC^{+} e C3646. O passo seguinte consistiu em avaliar a atividade antimicrobiana através da Concentração Mínima Inibitória (MIC). Assim, o quitosano que apresentou a menor MIC, com resultados reprodutíveis, foi o escolhido para a funcionalização com anticorpos específicos.

Conclui-se que apesar das CN sintetizadas de diferentes origens não apresentarem um efeito inibitório consistente contra a *L. monocytogenes,* as CN de Sigma mostraram ter um efeito promissor.

Palavras-chave: Anticorpos monoclonais; *Listeria monocytogenes*; Nanopartículas de quitosano; Propriedades antimicrobianas.

Abstract: Development of chitosan nanoparticles with antimicrobial activity against *Listeria monocytogenes.*

Listeria monocytogenes is one of the most dangerous food pathogens due to its resistance to adverse environmental conditions, and based on severity, listeriosis was the most severe, with the highest rate of hospitalization and mortality among the four most common zoonotic diseases reported in the Union European in 2019. Antibiotics have been essential to sustaining human and animal health. The WHO considered the level of antibiotic resistance in the last decade to be a serious threat to global health.

Thus, the use of natural compounds with antimicrobial activity is of interest, so in this work our attention was focused on chitosan, as it is FDA approved as Generally Recognized As Safe (GRAS) and has antimicrobial properties. The synthesis of chitosan nanoparticles (CN) has greater availability of interaction with other molecules and bacterial cells, as well as being able to react in acidic or basic pH. The antibacterial activity of CN is unspecific and therefore these NPs (nanoparticles) were functionalized with monoclonal antibodies (mAb) to improve its active targeting with the target pathogen, i.e *L. monocytogenes*. In this project, mAb C11E9 was used, which binds strongly to the bacterial cell wall of *L. monocytogenes*.

This project aims to evaluate the antimicrobial properties of CN synthesized from different sources and try to improve their properties against *L. monocytogenes*, functionalizing them with bioactive molecules.

For nanoparticle fabrication, the Ionic Gelation Protocol was used and three low molecular weight chitosans were selected, Sigma, HMC$^{+,}$ and C3646. The next step was to evaluate the antimicrobial activity through the Minimum Inhibitory Concentration (MIC). Thus, the CNs that presents the lowest MIC, with reproducible results, were chosen to be functionalized with mAb C11E9.

It is concluded that although the CN synthesized from different origins did not show a consistent inhibitory effect against *L. monocytogenes,* the CN from Sigma showed a promising effect.

Keywords: Antimicrobial properties; chitosan nanoparticles; *Listeria monocytogenes*; monoclonal antibodies.

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Abbreviation List

CDC- Centers for Disease Control and Prevention

STEC- Shiga Toxic-producing Escherichia coli

WHO- World Health Organization

L. monocytogenes- *Listeria monocytogenes*

EU- European Union

EFSA- European Food Safety Authority

CFU- Colony Forming Unit

RTE- Ready-To-Eat

ECDC- European Center for Disease Prevention and Control

EO- Essential Oil

GRAS- Generally Recognized As Safe

FDA- Food and Drug Administration

-$NHCOCH_3$- Acetamide group

-NH_2- Amino group

-NH_3^+- Protonated amino group

CNs- Chitosan Nanoparticles

NPs- Nanoparticles

LMW- Low Molecular Weight

EDL- Electrical Double Layer

MAb- Monoclonal Antibody

Ig- Immunoglobulin

SS- Sodium Sulfate

DLS- Dynamic Light Scattering

MIC- Minimum Inhibitory Concentration

LB- Luria Bertani Broth

O.D- Optical Density

***E. coli* O157:H7-** *Escherichia coli* O157:H7

1. Introduction

1. Introduction

1.1. Foodborne Diseases

Foodborne diseases are one of the most important public health problems associated with the risk of the emergence of antibacterial resistance in the food chain. In the literature, it has been indicated that an increase in antibiotic-resistant bacteria has caused an augment in foodborne diseases, and involves larger segments of the population, especially the elderly and people with immune system deficiencies (1). Additionally, the CDC (Centers for Disease Control and Prevention), which coordinate the investigation of possible multistate foodborne illness outbreaks, indicated that most of these diseases were caused by *Campylobacter*, *Listeria*, *Salmonella*, and Shiga Toxin-producing *Escherichia coli* (STEC) (2,3). In the EU, outbreaks of diseases transmitted by the bacteria previously described affected in 2019, 318.969 people in total, while in Portugal 1376 people were affected (3). Furthermore, as estimated by World Health Organization (WHO) foodborne pathogens cause 600 million cases of foodborne illnesses and 420.000 deaths globally every year, where 30% of the deaths are in children under 5 years of age (4).

1.2. *Listeria monocytogenes*

Listeria monocytogenes (*L. monocytogenes*) are Gram-positive bacillary bacteria with flagella, facultatively anaerobic, and do not produce spores. *L. monocytogenes* is one of the most dangerous and deadly foodborne pathogens, due to its resistance to harsh environmental conditions (temperature, salinity, pH, etc.), and its ability to form biofilms.

This bacterium can be found in moist environments, soil, water, decaying vegetation, and can survive, and even grow, under refrigeration and other food preservation conditions. When people eat food contaminated with *L. monocytogenes*, they may develop a disease called listeriosis (5), and based on severity, listeriosis was the most severe zoonoses with the highest hospitalization and mortality rate, compared to the most common zoonotic diseases reported in the European Union (EU) (6,7), as can be seen in **Table 1**.

Table 1 – Hospitalization and reported fatalities from the most common foodborne illnesses in the EU, 2019 (3).

Diseases	Reported Cases	Hospitalized Cases	Proportion Hospitalized	Deaths	Fatality Cases
Campylobacteriosis	220639	20424	31.8 %	46	0 %
Listeriosis	2621	1233	92.1 %	303	17.7 %
Salmonellosis	87908	16626	42.6 %	139	0.2 %
STEC Infections	7801	1099	37.9 %	10	0.2 %

There is a wide range of symptoms associated with listeriosis. Depending on the severity of the illness, may last from days to several weeks. Mild symptoms may include fever, muscle aches, nausea, vomiting, and diarrhea. If the most severe form of listeriosis develops, symptoms may include headaches, confusion, balance loss, and convulsions. For the very young, the elderly, and the immune-compromised listeriosis can result in death (5). Over 90% of listeriosis cases are attributable to doses > 10 000 Colony Forming Units (CFU) per serving, average of 50 g, corresponding to a mean bacterial count of 200 CFU/g food at the time of consumption. Furthermore, one-third of the cases can be attributed to the growth of *Listeria* during the storage phase in the consumers' homes (8).

Listeriosis can be particularly dangerous for pregnant woman and their newborn babies, leading to serious complications with their pregnancy, including miscarriage. Babies born with a listeriosis infection may develop severe health complications, such as some neurological disease, which requires immediate medical attention and can lead to lifelong problems or result in death.

Listeriosis outbreaks have been linked to raw, or unpasteurized milk and cheeses, vegetables, fruits, raw or undercooked poultry, sausages, hot dogs, deli meats, raw or smoked fish, and other seafood. *L. monocytogenes* has also been found in raw pet food and in Ready-To-Eat (RTE) food which is not heated before consumption.

The food can be contaminated when harvested, processed, prepared, packaged, transported, or stored in contaminated environments and *L. monocytogenes* can be transmitted to people through these contaminated foods (5). Although this problem is closely related to the practices in the food industries, these companies are motivated to find sustainable alternative solutions to ensure the safety of the product distributed on the market. At the same time, consumers are strongly oriented towards the consumption of food with characteristics of safety and quality (6).

1.3. Antibiotics and Natural Compounds with Biocidal Activity

Antibiotics have been essential to sustaining human and animal health. The WHO considered that the level of antibiotic resistance reached in the last decade is a serious threat to global health, which causes dangerous infections that can no longer be treated with traditional antibiotics (9). The extensive use of antibiotics in agriculture for growth promotion and disease prevention has major repercussions on farm animal production, food, and human safety (1).

The report on antibiotic resistance from the European Food Safety Authority (EFSA) and European Center for Disease Prevention and Control (ECDC) also highlights that bacteria found in animals, food, and humans can exhibit resistance to widely used antibiotics, which results in a public health hazard, limiting effective disease treatment strategies. It is estimated that at least 700.000 deaths per year worldwide are attributable to antimicrobial-resistant strains and this number is predicted to increase to 10 million deaths per year by 2050 if the incidence of antimicrobial resistance continues to escalate (10). Furthermore, the use of synthetic antiseptics, and germicides, in food processing may also favor the development of resistant pathogens, where globalization plays an important role. Intercontinental movement of food creates new opportunities for resistant pathogens to be spread globally (1).

Meanwhile, the use of natural compounds with biocidal activity against foodborne illnesses can be of interest. Previous studies showed particular interest in the application of plant essential oils (EO) against *L. monocytogenes*. The antibacterial effects of thyme oil inhibited the growth of this bacterium at low concentrations and could be considered as preservative materials for some kinds of foods or as additives to protect them from *Listeria* contamination (11). Another natural compound with biocidal activity against *Listeria* is phenolic compounds which have been used in industry for food safety purposes and also have inhibitory effects on pathogenic microorganisms. Extracts from different fruits and vegetables with high phenolic compound content showed inhibitory activity against *L. monocytogenes* (12).

Thus, we have focused our attention on chitosan, due to its great interest in this sector, which is Generally Recognized As Safe (GRAS) and approved as a food additive by the Food and Drug Administration (FDA), possesses a polymeric structure composed of randomly distributed units of D-glucosamine and N-acetyl-D-glucosamine linked *via* β (1-4) glycosidic bond (12,13) **(Figure 1)**.

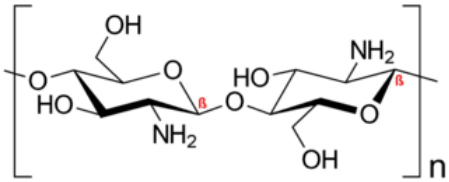

Figure 1 - Chemical structure of chitosan.

Chitosan, is an inexpensive cationic biopolymer obtained by deacetylation of chitin; a polysaccharide found in many kinds of crustacean shells as well as in the cell walls of fungi (13). Hence, this biopolymer is economic and the most abundant on Earth, after cellulose. During the deacetylation reaction, the acetamide groups (-$NHCOCH_3$) of chitin are replaced by amino groups (-NH_2), giving rise to chitosan **(Figure 2)**.

Chitosan has several advantages, including lack of toxicity, exceptional biocompatibility and biodegradability properties, antibacterial activity, and antioxidant effects (15). These have made it useful in several different industries such as medical, food, agriculture, textile, cosmetics, and other industries.

Chitosan exhibits a broad spectrum of antimicrobial, probably by the intracellular leakage *via* binding of the positively charged chitosan (-NH_3^+ groups) to the negatively charged bacterial surface. This interaction leads to a modification in membrane permeability, causing cell death. However, the antimicrobial activity of chitosan is limited only at an acidic pH, where the amino groups can be converted into their protonated and soluble form (-NH_3^+), and these groups are responsible for interacting with the external structures of microorganisms and destabilizing them (9).

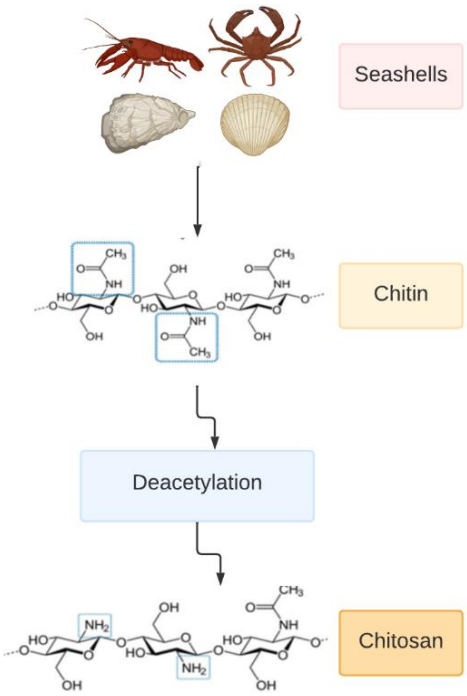

Figure 2 - Schematic representation of deacetylation of chitosan.

Thus, to attempt to enhance antimicrobial activity, it has been hypothesized that decreasing the particle size of chitosan, to nanoparticles, thus increasing the surface volume, may increase the binding on the bacterial surface, which can result in greater antimicrobial activity.

The unique characteristics of NPs can provide superior capabilities compared to their original counterpart, because the properties of the materials themselves remain relatively constant, however, as their size decreases, the surface volume increases, thus providing the nanoparticles with certain remarkable properties, as there are more particles to interact with the bacterial surface, that is, they have greater availability of interaction with other molecules or bacterial cells. The synthesis of nanoparticles uses Sodium Sulfate (SS) as a crosslinking agent, and this pairing of SS with chitosan provides the nanoparticles with an amphoteric character, that is, they are capable of reacting at an acidic or basic pH. Thus, the nanoparticles formed by this method called Ionic Gelation, are stable and have a positive surface charge (9,16).

The antimicrobial effect of CN may be due to the electrostatic interaction. Gram-positive bacteria have on their surface several glycolipids and polymers with negative charges such as lipoteichoic acid and peptidoglycan, which can interact with chitosan through - NH_3^+ groups. Electrostatic interaction, causes a destabilization of the cell wall, changing the permeability of the membrane, causing an osmotic imbalance and an efflux of cations occurs (chelating property), as well as, other intracellular substances. Consequently, these particles can also bind to DNA, inhibiting its replication, transcription, and enzyme production, causing cell death **(Figure 3)**.

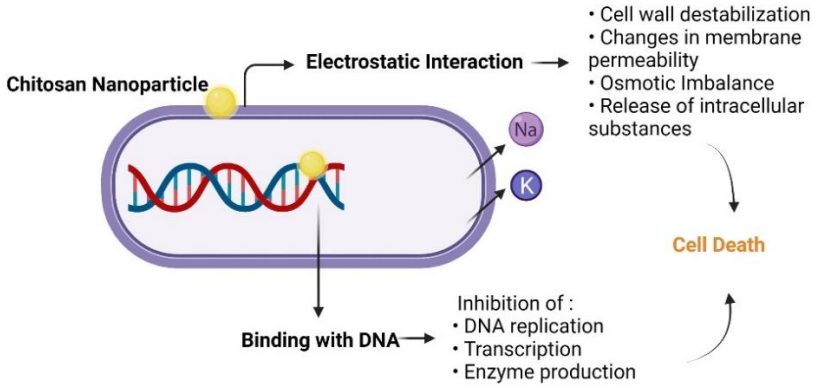

Figure 3 - Antibacterial mechanisms of chitosan nanoparticles.

Several parameters affect the antibacterial properties of CN, these include the molecular weight, particle size, degree of acetylation, and zeta potential.

The molecular weight of chitosan depends on its origin and method of preparation and influences the antibacterial activity. The bactericidal power of CN increases with decreasing molecular weight (14). Using low molecular weight (LMW) chitosan, significantly smaller particles can be obtained and may exhibit higher antimicrobial activity.

The degree of acetylation, is a parameter related to some biological properties of chitosan by influencing cell adhesion and is of fundamental importance for its performance as a biomaterial like inter and intramolecular interactions.

Previous studies showed that the higher the degree of acetylation, the lower the cell adhesion (14). This is because, as mentioned earlier, chitosan is mainly produced by deacetylation of chitin, so the lower the degree of acetylation, the more $-NH_3^+$ groups there will be in the solution and consequently may exhibit higher antimicrobial activity.

Zeta potential is a physical property that is exhibited by any particle in suspension and is a vital parameter for stability in aqueous nanosuspensions. Thus, the liquid surrounding the particle exists in two parts, a Stern Layer (inner region) where the ions are strongly bound, and ions with the opposite charge build the Diffuse Layer (outer region), where they are less firmly associated, and together form the so-called, Electrical Double Layer (EDL). Within the Diffuse Layer, there is a notional boundary, in which the ions and particles form a stable entity, the potential at this boundary is the zeta potential (17) **(Figure 4)**.

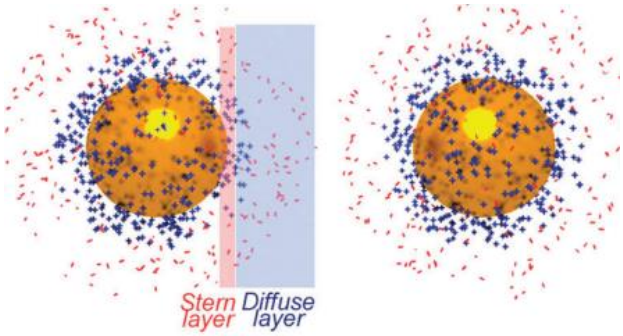

Figure 4 - Stern and Diffuse layer of particles (18).

The zeta potential's magnitude indicates the colloidal system's potential stability and indicates the degree of electrostatic repulsion between adjacent particles in a dispersion. If the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and therefore will not tend to come together, thus, colloids with high zeta potential are electrically stabilized. The general dividing line between stable and unstable suspensions is generally taken at either +30 or -30 mV. Particles with zeta potential higher than +30 mV or lower than -30 mV are normally considered stable (17).

In the case of chitosan, as it has a polycationic nature due to protonation of the $-NH_3^+$ groups, the zeta potential value is always positive, and studies have shown that CN with higher zeta potential values had greater effects on the inhibition of bacterial growth, because, higher zeta potential values makes nanoparticles to attach better to the bacterial cell membrane (19).

1.4. Antibodies

To attempt to improve the antimicrobial active targeting of CN against *L. monocytogenes*, antibodies were used, also called Ig (immunoglobulins). These molecules are produced by plasma cells, specifically lymphocytes B, in response to an antigen. There are two types of antibodies, polyclonals, and monoclonals. Polyclonals are a heterogeneous group of antibodies that each recognize a different epitope of the same antigen and are produced as a result of an immune response to an antigen that generally involves the activation of multiple B cells, all of which target a specific epitope of that antigen. On the other hand, monoclonals are a group that each recognize the same epitope of an antigen and are obtained by cloning a single parent B lymphocyte in response to a specific pathogen, as well as, these antibodies bind to the same epitope of that antigen.

In short, the main difference between them is in their origin, monoclonals antibodies are identical because they are produced from a single B lymphocyte cell, so they not only recognize the same antigen, but also bind to the same epitope, while, the polyclonals are produced from a mixture of lymphocytes and recognize multiple epitopes of the same pathogen (20). The epitope is the antigenic determinant, that is, the smallest portion of the antigen, in other words, it is the specific binding site that is recognized by the antibody.

Ig are typically Y-shaped and are composed of two light chains and two identical heavy chains. At the functional level, they have two main regions, the variable and the constant, the first being responsible for antigen recognition and the second having effector properties **(Figure 4)**. Ig can be divided into five different classes based on differences in amino acid sequences in the constant region of heavy chains and they are called isotopes (IgG, IgM, IgA, IgD, and IgE), which perform different functions contributing to an immune response according to each type of antigen. Classes of Ig can be divided into subclasses, each subclass plays a slightly different role in protecting the body from infection (IgG1, IgG2, IgG3, and IgG4) (21–23).

Figure 5 - The Ig heavy chain domains are shown in blue, while light chains are shown in green. The foot of the Y is called the constant fraction (Fc) and the arms (Fab) are the variant fraction, through which the antigen is bound.

In this project, monoclonal antibodies (mAb) were used, and these can act through several mechanisms, mediated by any of their regions, variables, or constants. A property of the most important functions is that mediated by antibody variable domains which consist of highly selective binding of the epitope on the target antigen, but often, the binding of an antibody/antigen has no direct biological effect. Significant biological effects are a consequence of the effector functions of antibodies, such effector functions include binding to various cell types, such as phagocytic cells, lymphocytes, platelets, and basophils that have receptors that bind Ig, and this connection can activate the cells and start to perform some functions, such as cytotoxicity and cellular phagocytosis.

The constant domains of antibodies perform functions of this type, and determine the functional characteristics of the antibody and effector activity (21,24).

In this research, mAb C11E9 was used, as previous studies showed that through the enzyme immunoassay (ELISA), which allows the detection of specific antibodies, it was found that 41 strains of *L. monocytogenes* tested, gave a positive reaction to this mAb. It was also possible to demonstrate that *L. monocytogenes* appear to express the majority of the C11E9 reactive protein of 174 kDa with κ light chains, which tightly bind to the bacteria cell wall. The C11E9 produces type G (IgG; gamma heavy chains), this isotope is found in greater amounts in our body and predominates in secondary responses, also called effector activities; and has IgG2 (gamma 2 heavy chains). IgG2 antibodies predominantly fight the polysaccharide (capsule) coating of certain disease-causing bacteria (25,26).

2. Objective

2. Objective

This project aims to evaluate the antimicrobial properties of chitosan nanoparticles synthesized from chitosan of different origins and attempt to improve their properties against *L. monocytogenes* by functionalizing them with specific mAb C11E9 for the recognition of this pathogen.

3. Materials and Methods

3. Materials and Methods

Three LMW chitosans were selected for the fabrication of CN. The following table shows the types of chitosan chosen and their respective molecular weight and degree of acetylation.

Table 2 - The chosen chitosan used in this project and their respective molecular weight and degree of deacetylation.

Type of Chitosan	Molecular Weight (kDa)	Degree of Deacetylation
Sigma (27)	50-190	≥ 75 %
HMC^{+} (28)	80-200	≥ 92 %
C3646 (29,30)	90-190	≥ 75 %

3.1. Fabrication of CN: Ion Gelation Protocol

The particles were generated following an ion gelation protocol: a 2 % (2 g) chitosan solution was prepared in 2 % (2 mL) acetic acid and 1 % (1 g) Tween 80, in a final volume of 100 mL. The addition of 0.5 % (5 mL) of the cross-linker, sodium sulfate (SS-10 %), was done drop by drop with constant stirring and sonication (Sonifier Sound Enclosure-Branson) at 60 W for 20 min. The newly formed CN solution was sonicated for an extra 25 min. Once completed, the CN was transferred to a dialysis membrane (OrDial D14 – MWCO 12000-14000 – Orange Scientific) and placed in a beaker with milli-Q water. The water was changed every hour for three hours, then left overnight and changed one last time on the following day to make a total of four water changes. The representation of the cross-linking between CN with SS is shown in **Figure 5.** To determine the average concentration of dry weight of chitosan nanoparticles, three independent 1 mL aliquots were dehydrated overnight at 62°C (**Table 3**).

Table 3 - The final concentration of the CNs synthesized from different chitosan.	
CNs	**Final Concentration (g/mL)**
Sigma	0.02
HMC^{+}	0.015
C3646	0.017

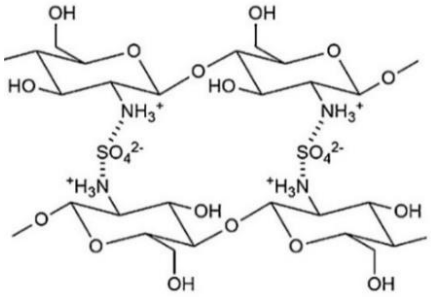

Figure 6 - Schematic representation of the cross-linking of the chitosan by sodium sulfate (9).

The particle size and the zeta potential were measured and the Nanoparticle Analyzer (SZ-100, Horiba Scientific), was used. Dynamic Light Scattering (DLS), is a technique classically used for measuring the size of particles dispersed in a liquid, DLS measures Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles by the solvent molecules that surround them, the larger the particle, the slower the Brownian motion will be (31). First divided into two tubes of each CNs, one of the tubes was sonicated on the day it was made and kept in the refrigerator at 4°C until used in the experiments described later; more time in the refrigerator, implies that the particles aggregate more, forming a type of gel; for now on, Sigma 1x Sonic, HMC^{+} 1x Sonic and C3646 1x Sonic. The second tube was sonicated just before measuring; so for now on, Sigma 2x Sonic, HMC^{+} 2x Sonic, and C3646 2x Sonic (**Table 5**). The purpose of dividing the tubes of each chitosan was to compare how the particles behave when they stay for some time in the refrigerator and then after sonicating.

Afterward, to measure the size of the particles and the zeta potential for each chitosan, it was important to dilute the samples at 250x, so 1 mL of Milli-Q water and 20 µL of each CNs were prepared (500x), and then each sample was diluted with 1 mL Milli-Q water and 200 µL of the previous sample (250x), and through DLS analysis the followed values were obtained (**Table 6**).

3.2. Evaluation of antimicrobial activity: MIC

The following step was the evaluation of antimicrobial activity, which was assessed by the Minimum Inhibitory Concentration (MIC). MIC is defined as the lowest concentration of an antimicrobial ingredient or agent that is bacteriostatic (prevents the visible growth of bacteria) (32).

A fresh culture of *L. monocytogenes* was prepared by inoculating one single colony in 4 mL of LB (Luria Bertani broth, PanReac AppliChem, ITW Reagents) and grown overnight at 37°C. This culture was transferred (50 µL; 1:200 dilution) to fresh LB and incubated at the same temperature with constant agitation (120 rpm) until an Optical Density (O.D) at 600 nm of ± 0.5 was reached (10^8 CFU/mL), which was diluted until reaching a concentration of 10^6 CFU/mL. Then, 100 µL of the bacterial culture, were transferred to ten tubes, in a final volume of 10 mL of LB rendering a final bacterial concentration of 10^4 CFU/mL. First, 100 to 1000 µL of Sigma solution were added, which correspond to a concentration of 0.2 and 2.0 mg/mL respectively. Furthermore, previous studies showed that nanoparticulate chitosan under these conditions inhibits the growth of *Escherichia coli* O157:H7 (*E. coli* O157:H7), so we used this bacterium for comparison (1). Afterward, the results will be presented and discussed.

3.3. Functionalization of CN with antibodies

Subsequently, the CN that presents the lowest MIC, with reproducible and constant results, was chosen for the functionalization with antibodies in order to try to improve its antimicrobial active targeting, as well as, specify its action, so a specific mAb C11E9 against *L. monocytogenes*, was used.

Thus, three different concentrations of C11E9 were used, 0.02, 0.008, and 0.003 mg/mL (**Table 4**), each with 1.5 mL of the selected chitosan. Then, they were transferred to a dialysis membrane (Dialysis Tubing – MWCO 10K, 16 mm – Thermo Scientific) and placed in a beaker with 50 mL of Phosphate Buffer (PB, 10 mM) for 2h, due to the presence of Sulfamethoxazole in MAb, which can interfere with its antimicrobial activity, as it is an antibiotic. Once completed, they were transferred to the respective Eppendorf tubes and placed on the rotary shaker overnight in the cold room at 4°C, the next day the samples were ready for the experiment. Afterward, to measure the size of the particles for each concentration of antibody, 1 mL of Milli-Q water and 50 µL of each sample were prepared, and then each one was diluted with 1 mL Milli-Q water and 200 µL of the previous sample, through DLS analysis the followed values were obtained (**Table 7**).

Table 4 - The volume and respective concentration of mAb C11E9 with 2.1 mg/mL.

Volume (µL)	Final Concentration (mg/mL)
12	0.02
6	0.008
2	0.003

4. Results and Discussion

4. Results and Discussion

To determine the MIC of CNs, Sigma was the first to be used. As expected, and by previous studies (9), it was observed that CNs effectively inhibited the growth of *E. coli* at a final concentration of **1.2 mg/mL**, thus this value is considered the **MIC**, as can be observed in **Figure 7**.

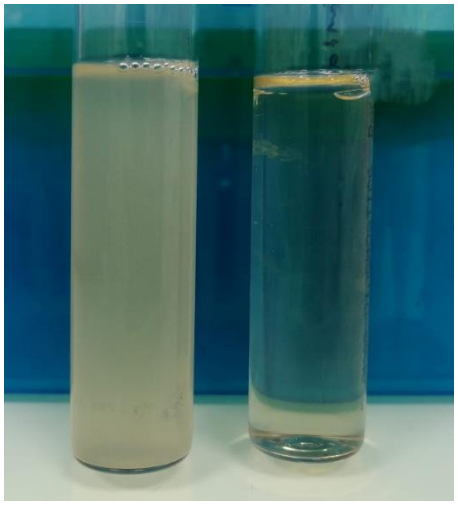

Figure 7 - Inhibition of the growth of E. coli bacteria. The tube on the left side is 1 mg/mL and the one on the right side is 1.2 mg/mL of Sigma.

L. monocytogenes, on the other hand, was fully inhibited even at the lowest concentration of CN tested, 0.2 mg/mL, as can be seen in **Figure 7.**

Figure 8 - Total inhibitory action of CN on the growth of L. monocytogenes. From left to right: 0.2, 0.4, 0.8, 1.6 and 2.0 mg/mL of Sigma.

One of the main reasons why CN has antimicrobial activity is the presence of protonated amine groups, that interact with the negative charges of the bacterial cell wall and, as Gram-positive bacteria, *L. monocytogenes*, have several glycolipids and polymers on their surface with highly negative charges such as lipoteichoic (the main constituent of the cell wall) and peptidoglycan (a constituent of the outer membrane), that interact with NH_3^+ groups, through electrostatic interaction. In conclusion, perhaps this is why CN has more inhibitory action against *L. monocytogenes* than *E. coli* Gram-negative bacteria).

Thus, the tested concentration range was reduced. In this sense, the concentration covered the range from 0.02 to 0.2 mg/mL. In these assays, *E. coli* with 0.2 and 2.0 mg/mL of CN, was used as negative and positive controls, respectively (**Figure 9**). **Figure 10** shows the inhibitory action of Sigma against *L. monocytogenes* and its MIC.

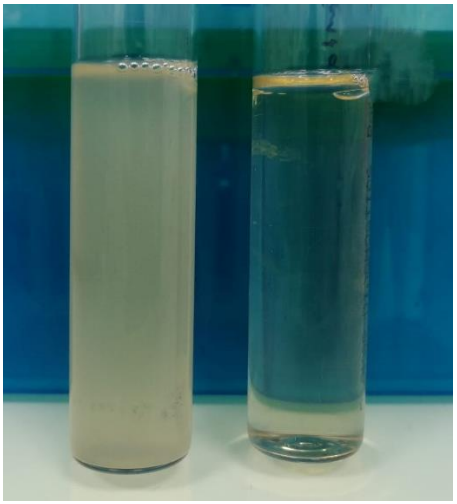

Figure 9 - Negative and positive controls with E.coli with 0.2 and 2.0 mg/mL of Sigma, respectively.

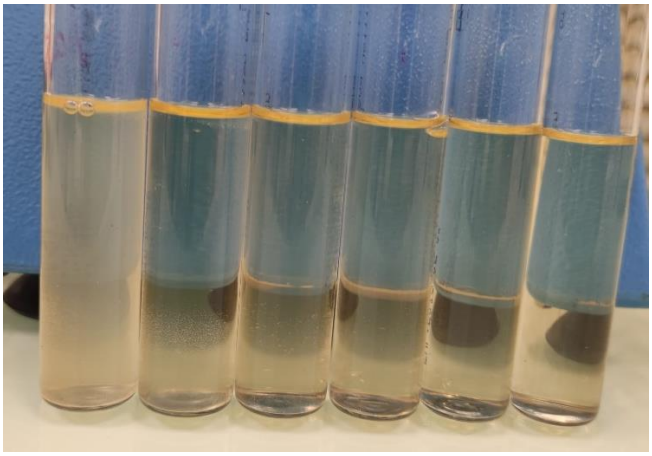

Figure 10 - Inhibitory action of Sigma against L. monocytogenes. From left to right: 0.02, 0.04, 0.08, 0.12, 0.16, and 0.2 mg/mL of Sigma.

Attending to the results, the **MIC** for *L. monocytogenes* was determined to be **0.04 mg/mL** for Sigma chitosan. The procedure was repeated and the same results were obtained.

The second type of chitosan used was HMC^{+} and the last procedure was performed. Figure 11 shows positive and negative control with *E. coli* and Figure 12 represented the inhibitory action of CN against *L. monocytogenes* and MIC.

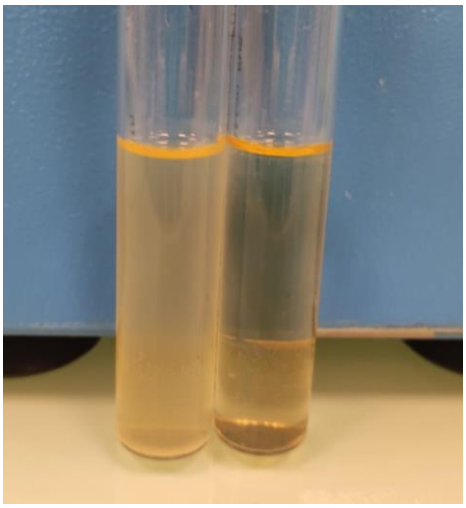

Figure 11 - Negative and positive controls were made with E.coli with 0.15 and 1.5 mg/mL of HMC+.

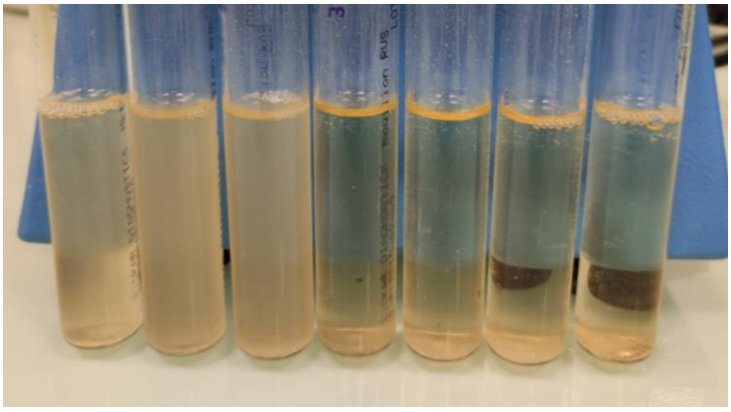

Figure 12 - . Inhibitory action of HMC+ against L. monocytogenes. From left to right; 0.02, 0.03, 0.05, 0.06, 0.09, 0.12, 0.15 mg/mL of CN.

According to the results**, 0.06 mg/mL** was determined to be the **MIC** against *L. monocytogenes* for HMC^+ chitosan. The procedure was repeated and no reproducible results were obtained, as can be seen in **Figure 13**, HMC^+ was inhibitory at 0.05 mg/mL, but growth was verified at 0.08 mg/mL, and from 0.09 mg/mL onwards there was second growth inhibition, and **Figure 14**, HMC^+ was inhibitory at 0.05 mg/mL, but growth was observed at the two highest CN concentrations, 0.14 and 0.15 mg/mL.

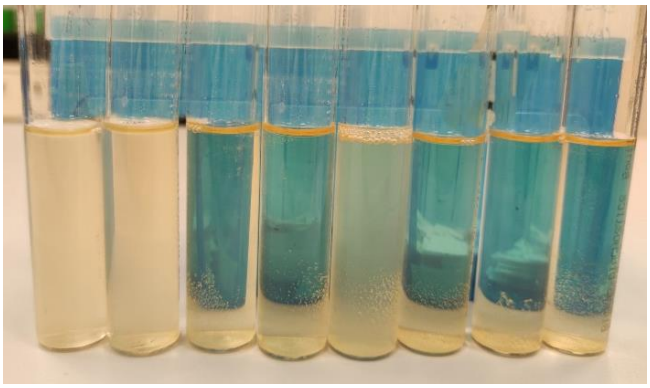

Figure 13 - Second growth inhibition of L. monocytogenes with 0.09 mg/mL of HMC+ chitosan. From left to right: 0.02, 0.03, 0.05, 0.06, 0.08, 0.09, 0.12, 0.15 mg/mL..

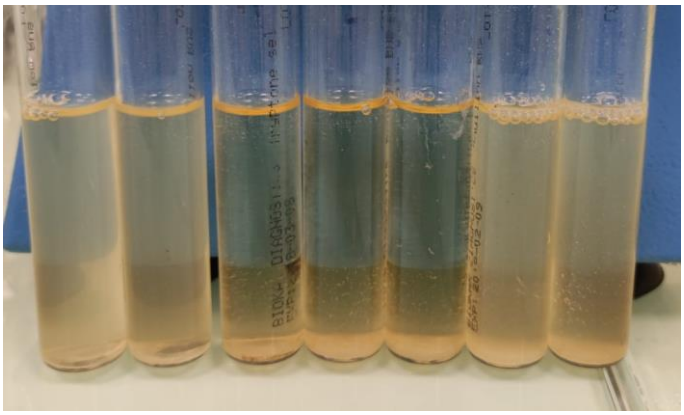

Figure 14 - Inhibition of the growth of L. monocytogenes at 0.05 mg/mL but second growth was seen at the two highest CN concentrations, 0.14 and 0.15 mg/mL. From left to right; 0.02, 0.03, 0.05, 0.06, 0.09, 0.12, 0.14, 0.15 mg/mL.

The last type of chitosan used was C3646 and the previous experiment was performed. In these assays, *L. monocytogenes* with 0.2 and 0.4 mg/mL of Sigma, was used as negative and positive controls, respectively (**Figure 15**). In **Figure 16**, the inhibitory action against *L. monocytogenes* of the CN and its MIC is shown.

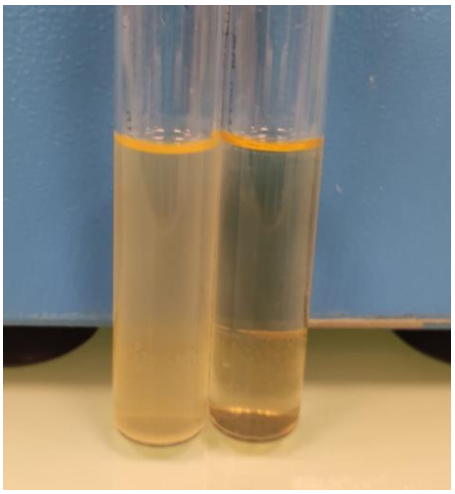

Figure 15 - Negative and positive controls with L. monocytogenes with 0.2 and 0.4 mg/mL of Sigma, respectively.

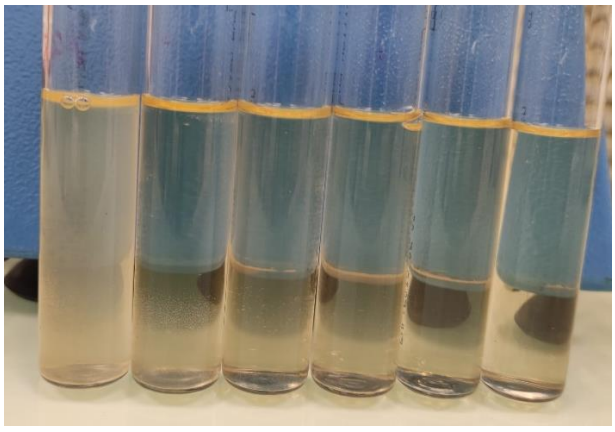

Figure 16 - Inhibitory action of C3646 against L. monocytogenes. From left to right: 0.02, 0.03, 0.07, 0.10, 0.14, and 0.17 mg/mL of C3646.

Attending to the results, the **MIC** for *L. monocytogenes* was determined to be **0.03 mg/mL** for chitosan C3646. The procedure was repeated and the same results were not obtained, as can be seen in **Figure 17**, the highest concentration of CN shows bacterial growth.

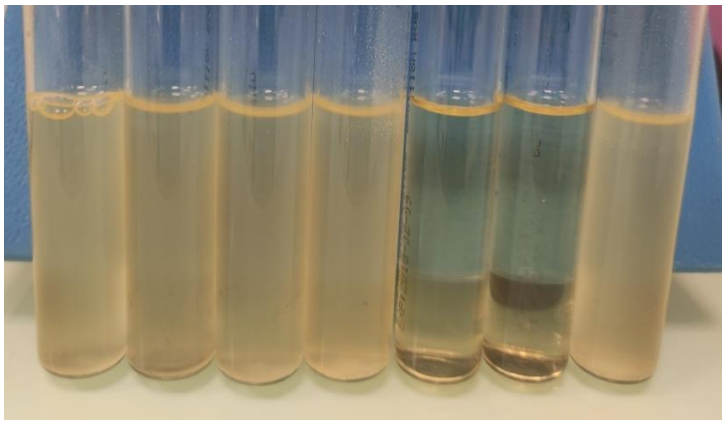

Figure 17 - Inhibition of the growth of L. monocytogenes at 0.14 and 0.15 mg/mL. From left to right 0.02, 0.03, 0.07, 0.10, 0.14, 0.15 and 0.17 mg/mL of C3646.

As previously stated, molecular weight, degree of acetylation, particle size, and zeta potential are important factors that affect the antimicrobial properties of CN (**Table 5** and **Table 6**).

Table 5 - Molecular weight, degree acetylation and MIC of each CN.

Type of CN	Molecular Weight (kDa)	Degree of Deacetylation	MIC (mg/mL)
Sigma	50-190	75 %	0.04
HMC+	80-200	92 %	0.06
C3646	90-190	75 %	0.03

The antibacterial activity of CN increases with decreasing molecular weight (19).

Thus, although the CNs under study were of low molecular weight, the results obtained with the chitosan Sigma were considered the best among the different ones tested as having a low MIC of 0.04 mg/mL and the results were reproducible and constant.

The degree of deacetylation is a parameter that influences these biopolymer's inter and intramolecular interactions and, therefore, their properties. In previous studies, it has been shown that the higher the degree of deacetylation, the lower the cell adhesion (19). As Sigma and C3646 have a lower degree of deacetylation compared to HMC^+, 75%, and 92% respectively, it be can speculated that differences in their antimicrobial activities may be influenced by this parameter.

Previous studies reported that smaller particles had lower MIC values (0.04 mg/mL for Sigma, 0.06 mg/mL for HMC^+, and 0.03 mg/mL for C3646), indicating superior antibacterial effects (14). Thus, the next step was to measure the particle size and the zeta potential of each chitosan, through DLS analysis the followed values were obtained (**Table 6**). As has been said before, zeta potential is a vital parameter for stability in aqueous nanosuspensions. Studies have shown that CN with higher zeta potential values had greater effects in inhibiting bacterial growth (17).

Table 6 - Size (nm), zeta potential (mV), of each chitosan with five samples.

	Sigma 1x Sonic	**HMC^+ 1x Sonic**	**C3646 1x Sonic**
Size ± SD (nm)	355.82 ± 142.58	789.98 ± 356.37	402.56 ± 95.60
Zeta ± SD (mV)	51.84 ± 2.06	57.58 ± 1.72	45.88 ± 0.49
	Sigma 2x Sonic	**HMC^+ 2x Sonic**	**C3646 2x Sonic**
Size ± SD (nm)	174.13 ± 53.16*	533.76 ± 456.23	880.9 ± 599.16*
Zeta ± SD (mV)	36.8 ± 14.80**	38.04 ± 16.16	27.03 ± 14.72**

*This value was calculated with 4 samples as the other one was an outlier.
**This value was calculated with only 3 samples as the other two were an outlier.

Subsequently and according to the results, Sigma CN is the one that presents the lowest MIC with reproducible results. It also presents stability as well as its zeta potential, and thus it was chosen for the functionalization with mAb C11E9.

Using the same procedure as above and knowing that the MIC of Sigma is 0.04 mg/mL, firstly only two concentrations, 0.02 and 0.04 mg/mL of Sigma CN were used for each antibody concentration (0.02, 0.008, and 0.003 mg/mL) with a final volume of 10 mL of LB, which was inoculated with 100 µL of *L. monocytogenes* culture, rendering a final bacterial concentration of 10^4 CFU/mL (**Figure 19-21**). In these assays, *L. monocytogenes* with 0.02 and 0.04 mg/mL of Sigma (0 µL Ab) was used as a negative and positive control, respectively, as can be seen in **Figure 18**.

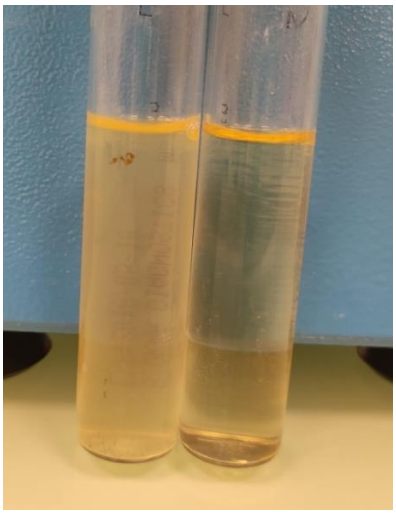

Figure 18 - Negative and positive controls with L. monocytogenes with 0.02 and 0.04 mg/mL of Sigma.

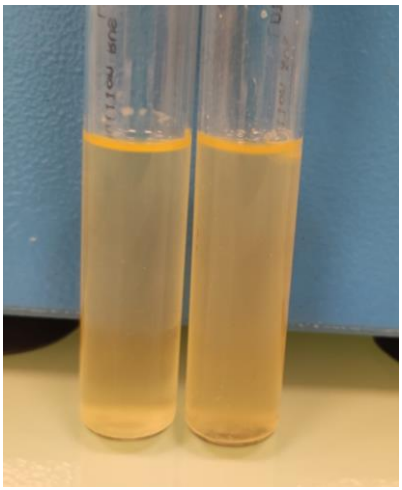

Figure 19 - . Growth of L. monocytogenes culture with 0.02 and 0.04 mg/mL of Sigma CN with 0.02 mg/mL of C11E9.

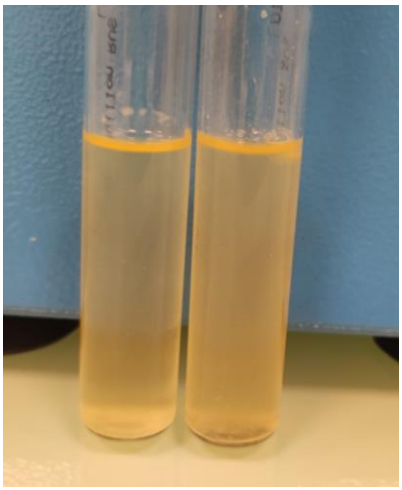

Figure 20 - Growth of L. monocytogenes cultured with 0.02 and 0.04 mg/mL of Sigma CN with 0.008 mg/mL of C11E9.

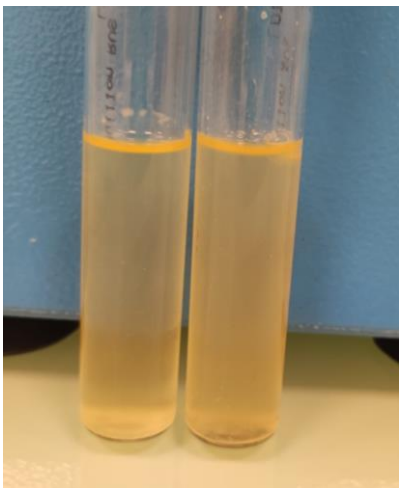

Figure 21 - Growth of L. monocytogenes culture with 0.02 and 0.04 mg/mL of Sigma CN with 0.003 mg/mL of C11E9.

According to the results, it can be concluded that it was necessary to increase the tested concentration of CN. In this sense, the concentration covered the range from 0.04 to 0.08 mg/mL. The same procedure was repeated and the results will be represented and discussed below.

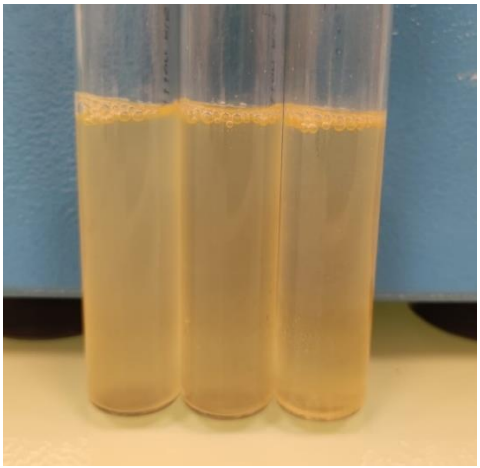

Figure 22 - Growth of L. monocytogenes cultured with 0.04, 0.06, and 0.08 mg/mL of Sigma CN with 0.02 mg/mL of C11E9. Left to right: 0.04, 0.06, and 0.08 mg/mL.

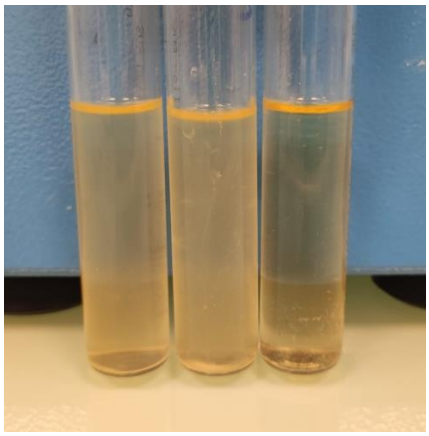

Figure 23 - Inhibition of the growth of L. monocytogenes at 0.08 mg/mL of Sigma CN with 0.008 mg/mL of C11E9. Left to right: 0.04, 0.06, and 0.08 mg/mL.

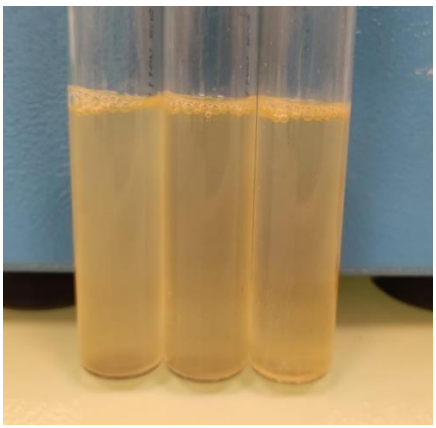

Figure 24 - . Growth of L. monocytogenes cultured with 0.04, 0.06, and 0.08 mg/mL of Sigma CN with 0.003 mg/mL of C11E9. Left to right: 0.04, 0.06, and 0.08 mg/mL.

The procedure was repeated and the same results were not obtained, as can be seen in the following figures.

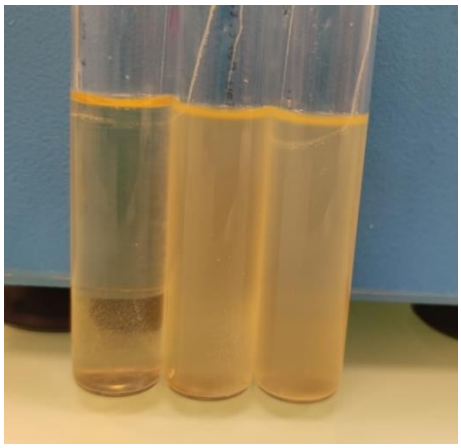

Figure 25 - L. monocytogenes growth inhibition with 0.04 mg/mL of Sigma CN and 0.02 mg/mL of C11E9. Left to right: 0.04, 0.06, and 0.08 mg/mL.

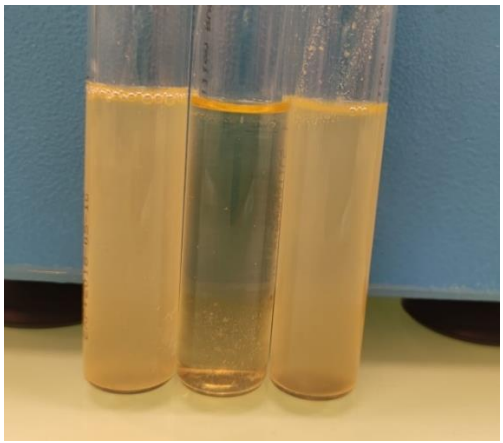

Figure 26 - . L. monocytogenes growth inhibition with 0.06 mg/mL of Sigma CN and 0.008 mg/mL of C11E9. Left to right: 0.04, 0.06, and 0.08 mg/mL.

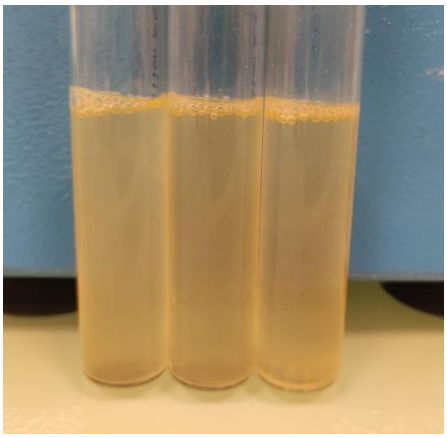

Figure 27 - Growth of L. monocytogenes cultured with 0.04, 0.06, and 0.08 mg/mL of Sigma CN and 0.003 mg/mL of C11E9. Left to right, 0.04, 0.06, and 0.08 mg/mL.

The following step was to evaluate the particle size of each concentration of antibody. As previously stated, particle size is one of the most important factors that affect the antimicrobial properties of CN, through DLS analysis the followed values were obtained (**Table 7**).

Table 7 - Size (nm) of each concentration of MAb C11E9 with five samples.

	0.02 mg/mL Ab	**0.008 mg/mL Ab**	**0.003 mg/mL Ab**
Size ± SD (nm)	56673.6 ± 18885.1	43294.6 ± 12258.7	39709.5 ± 32376.0

According to the results obtained, it can be hypothesized that the inconsistency of the antimicrobial activity displayed by the CN functionalized with different concentrations of antibody, may be associated with a significant increase in particle size, from 174.13 nm to 39709.5 nm. As mentioned earlier, a larger size implies a lower inhibitory effect, as its membrane-binding affinity decreases due to the decrease in the interaction of nanoparticles with the bacterial membrane.

5. Conclusion

5. Conclusion

According to the values and parameters, Sigma's chitosan can be considered to have a superior antibacterial effect over the others. Using lower LMW, significantly lower CN can be obtained and these particles are reported to have higher antimicrobial activity with constant and reproducible results. Thus, Sigma's chitosan can then be applied in studies to assess its applicability in different fields, such as treating or preventing animal diseases, introducing them, for example into their food, or even eliminating other pathogens from food, such as, in RTE salads, which do not undergo heat treatment before consumption.

With the functionalization of Sigma's chitosan with bioactive molecules, in this case with specific monoclonal antibodies, mAb C11E9, it was verified that there was an inconsistency in the results, perhaps, not only to the large size of the particles, but also, to the fact that the antibodies, randomly occupy the surface of the particle of chitosan, causing the inconsistency of the antimicrobial activity presented by CN Sigma with different concentration of mAb C11E9.

Furthermore, the development of novel bioengineering methods to minimize the spread of bacteria in food processing chains and other strategies to fight against food bacteria should be considered and improved. It is also recommended the implementation of protocols for dispersion of CN for their functionalization with antibodies, since it is speculated that a good dispersion of the particles, the antibodies would not occupy the surface of the particles randomly and even promising results could be obtained. It is expected the continuity of studies in this sense in order to consolidate scientific knowledge.

6. References

6. References

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