University of Stavanger

Faculty of Science and Technology

Nofima

Stavanger

MASTER'S THESIS

Study program/ Specialization: Master of Science in Biological chemistry	Spring semester, 2017 Open access
Writer: Miriam Høie	Miriam Høie (Writer's signature)
Faculty supervisor: Peter Ruoff External supervisors: Jan Thomas Rosnes and Morten Sivertsvik	
Thesis title: Antibacterial effect of chitosan in relation to active food packaging	
Credits (ECTS): 60	
Key words: Chitosan, food packaging, *Escherichia coli*, *Staphylococcus aureus*, BioScreen, ISO22196, inhibition	Pages: 54 + appendix: 7 Stavanger, June 15th 2017

Front page for master thesis
Faculty of Science and
Technology
Decision made by the Dean October 30th 2009

Abstract

Food safety has a high priority in the food industry. In order to be able to give the consumers safe products, the producers continuously work with methods to inhibit growth of bacteria in the foods during storage. Recently, the industry has paid attention to the use of active packaging methods that contain antimicrobial substances. Among these, chitosan has been widely investigated. In this study, potential packaging materials containing chitosan was tested against the Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) and the Gram-negative bacterium *Escherichia coli* (*E. coli*). One of the packaging materials was used to make trays in which smoked salmon was packaged, and the fish was tested during storage to see if there was a detectable effect of the active packaging material.

The method ISO 22196:2007 was used to compare the antibacterial effect of different chitosan-containing films when they were tested against the same volume of bacteria, with a known contact area between the bacteria solution and the active film. This method had a lot of elements affecting the results, and also many uncertainties. The method was unstable, giving results that were invalid according to the standard. However, there seemed to be some antibacterial effect from the chitosan-containing films. The film containing MB as plasticizer, 8% polyethylene glycol and 2% modified chitosan with nanoclay C30B caused the largest log reduction of *E. coli*, log 3.67. Against *S. aureus* the films containing MB as plasticizer, polyethylene glycol and 1% rosehip-modified chitosan gave best results, with a log reduction of 4.48.

In order to gain more reliable results, a method was used where chitosan was tested in a liquid bacteria solution. In this method, different amounts of chitosan film containing 5% chitosan and chitosan powder was added to the bacteria solution and incubated at different temperatures with regular sampling during incubation. The best effect of the chitosan film was seen when the samples had an initial bacterial concentration about log 5 cfu/ml with incubation at 10°C. Here, a clear effect was found against both of the tested bacteria; *E. coli* decreased below the detection limit after five days and *S. aureus* after six days for the samples containing most of the chitosan film. The results from incubation with chitosan powder were similar to those from testing of the film, but the bacterial counts decreased below the detection limit earlier in the incubation. *E. coli* counts were below the detection limit after four days, and *S. aureus* counts after three days for the samples with most chitosan, when having a start concentration of bacteria about log 5 cfu/ml with incubation at 10°C. These results strengthens the theory that chitosan is less available inside a rigid film structure than it is in the form of chitosan powder.

As the results from the liquid tests were promising, chitosan colloid was tested by comparison of growth curves with and without chitosan. The chitosan powder was solved in acetic acid, and this solution was then tested against the aforementioned bacteria by reading absorbance in bacteria solutions during incubation. The data was used to make growth curves, which made it possible to compare the bacterial growth between samples with and without chitosan. The acid tolerance of the bacteria became very limiting for the experiments, but at acid concentrations of 0.1% and 0.5% it was possible to detect an effect of the chitosan compared to the control samples. However, the effect might not be caused by the chitosan alone, but rather of a combination of chitosan together with the lowered pH.

The reason of testing the chitosan films and powder was to see if chitosan-containing packaging materials could be used on foods to enhance food safety. Because some of the test methods gave good results, an experiment was conducted on smoked salmon. The salmon was packaged in 100% $N_{2\,(g)}$ in a tray containing 1% chitosan. This was a storage experiment, and sampling was performed during storage for 41 days. Compared to the control sample, packaged in a HDPE tray, the salmon packaged in chitosan trays had the same bacterial numbers in the beginning of the experiment, but towards the end the bacterial numbers in the sample packaged in PLA-chitosan tray increased to log 7 cfu/g, while the control sample ended at a bacterial number of log 5 cfu/g. This shows that the bacteria in the fish samples grew despite the presence of chitosan, which can be caused by the chitosan not being available for the bacteria, but it can also be that the trays should have had a higher chitosan concentration.

Acknowledgements

This research thesis was conducted to meet the requirement of Master of Science degree in the Faculty of Natural science and Technology in University of Stavanger. The research was performed at Nofima, Måltidets Hus, Stavanger.

First, I would like to thank my thesis supervisors Jan Thomas Rosnes, Morten Sivertsvik and Peter Ruoff. The doors to their offices were always open when I met problems or had questions regarding my research or writing. They allowed me to work independently, yet they always gave me advice when I needed it. I would especially like to thank Jan Thomas Rosnes for his excellent guidance during both laboratory work and equipment, and also during writing. I am also very grateful to Morten Sivertsvik for good advices regarding practical work and also during writing.

I would also like to thank the scientists and technicians at Nofima Stavanger for their support during my research, especially Leena Shinde and Karin Tranøy who worked with me and assisted me in the laboratory experiments.

Finally, I want to express my greatest thanks to my parents and to my living partner for providing me with full support, care and encouragement throughout my years of study and during the process of researching and writing this thesis. This would not have been possible without you.

Thank you.

Table of contents

1 Introduction

In the food industry, food safety has a high priority. In order to be able to guarantee the consumers a safe product at time of consumption, producers continuously work to inhibit the growth of microorganisms in the food products (Alboofetileh, Rezaei, Hosseini, & Abdollahi, 2014). They hope to develop new packaging methods that actively inhibit the microbial growth, and researchers have worked with different theories and materials for a long time. Most of the methods that are in use today are characterized as synthetic preservation techniques (Zhang, Liu, Wang, Jiang, & Quek, 2016), and they often include use of chemicals. It has been questioned if this way of preservation represent a health hazard ("Food Safety: What you should know," 2015), and an alternative is wanted by many.

As the focus on environmental challenges increases, the request for a natural, biodegradable packaging film that will reduce the use of synthetic materials is increasing (Rhim, Hong, & Ha, 2009).Today the food is packaged in materials that are made from petrochemical sources. In order to enhance the food safety and the shelf life of the packaged product, the food industry have paid attention to the possibility of developing degradable packaging films that hold antimicrobial properties (Guirguis, Abd Elkader, & Nasrat, 2013). As an alternative to the chemical preservatives, there are many natural antimicrobials that can easily be either incorporated into the packaging material, or applied inside the package. Many natural, active components have been tested, one of which is chitosan.

Chitosan is the product when chitin is deacetylated in an alkaline environment (Dutta, Tripathi, & Dutta, 2012; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Chitin is a natural biopolymer that is found naturally in the shells of crustaceans, in insects, and it is also produced by fungi (Al-Sagheer, Ibrahim, & Khalil, 2014; Portes, Gardrat, Castellan, & Coma, 2009; Sébastien, Stéphane, Copinet, & Coma, 2006). These sources of chitin are otherwise not utilized, meaning that production of chitosan-incorporated packaging films could reduce the amounts of waste from other industries.

The properties of chitosan itself have been investigated by numerous research groups in order to document the antimicrobial effect, and also to identify advantages and possible disadvantages of its use. In order to make a packaging film containing chitosan, a plasticizer is necessary. Polylactic acid (PLA) has been tested for this purpose in order to see what abilities PLA-chitosan films possess (Râpă et al., 2016). Much research has been done on alternative packaging materials, and possible solutions are tested regularly. These materials are often more expensive than the ones that are in use today, and the composition of the packaging material have to be tested to find a material that has satisfying results when it is tested on foods, not just in laboratory solutions.

In this research, different chitosan-containing films were tested against the two foodborne pathogens *Escherichia coli* and *Staphylococcus aureus*. The films were tested on liquid bacteria solutions by use of three methods, and chitosan-containing trays were tested on smoked salmon in a storage experiment.

2 Theory

2.1 Consumer demands to new packaging solutions

Consumers have always payed attention to the quality of foods, desiring products that can be stored over time with a low level of deterioration. The focus on the environment has been constantly increasing, and several proofs have shown that humans affect the earth in a major degree than what have been known from earlier (Leceta, Guerrero, & De La Caba, 2013). In the industrialized countries, consumers are being more aware of the way their lifestyle affects the health than ever before (Alishahi & Aïder, 2012; G.-H. Wang, 1992). This makes them more critical to the contents of food they are buying, at the same time that they request foods that are packaged in materials that are natural, degradable and environmentally friendly (Bonilla, Fortunati, Vargas, Chiralt, & Kenny, 2013). As a consequence of this, there have been an increasing interest in developing packaging methods and materials that are degradable, at the same time that they contribute to improved food quality by controlling microbial growth (Leceta, Guerrero, & De La Caba, 2013; Massouda, Visioli, Green, & Joerger, 2012).

The awareness related to chemical food preservatives has also increased, and consumers request products that are “natural”. They want foods that have been mild processed, and they request products which contain natural preservatives instead of chemicals that might be carcinogenic or toxic (Han, Patel, Kim, & Min, 2014; Lucera, Costa, Conte, & Del Nobile, 2012; Massouda et al., 2012; Tassou & Nychas, 1995). Preservatives are necessary in order to obtain a good microbial quality of food, and to inhibit bacterial growth in the foods (G.-H. Wang, 1992). In addition to constant changes in the production and distribution, these demands from the consumers to the producers represent big challenges in order to maintain the quality and safety of foods (Han et al., 2014; Realini & Marcos, 2014).

2.2 Conventional food packaging

The consumers are one of the major driving forces towards development and change of packaging materials. Another driving force is the development in distribution and marketing. Factories are placed in countries where the production cost will be lowest, and the products are then distributed all over the world. These changes require different kinds of packaging materials, with different abilities. At present, the food is mostly packaged in plastic materials. These are efficient materials that keep the food separated from the surrounding microbiota, but they cause pollution of the environment, and hence they are no longer as satisfying for the consumers as they used to be before alternatives existed (Wickramarachchi, Samaratunge, Kaushalya, Rasangika, & Paranagama, 2016). A consequence of the consumer demands and the environmental concerns, is that the research on packaging methods has an increasing focus on packaging materials with a majority of natural components (Remya et al., 2016). This research focus also reflects the fact that the food industry wants an alternative to petrochemical-based packaging materials with an acceptable cost, that might reduce the amount of polluting waste from the food industry (Abdollahi, Rezaei, & Farzi, 2012; Realini & Marcos, 2014).

However, it is not only the environmental factors that are driving forces towards innovation. Foodborne pathogens are a continuous health hazard all around the world, and active packaging

systems that contain antimicrobial components can be useful to control the microbial growth in the foods that occurs after processing (Remya et al., 2016; Torlak & Sert, 2013). If this kind of packaging system can be used commercially, the shelf life of foods can be increased, resulting in a reduced amount of foods being wasted due to deterioration (Realini & Marcos, 2014).

2.3 Active packaging

Food packaging aims to protect and preserve foods, and it is important for the food producer in order to be able to ensure a safe and high-quality product at time of consumption (Youssef, El-Sayed, Salama, El-Sayed, & Dufresne, 2015). In order to prolong the time period of which the food quality is within the acceptable limits, active packaging can be used. There is no exact definition of active packaging, as it is not just a way of packaging. It can include numerous additives or other variations that in combination obtain food quality, either by adding them in the packaging material itself or by application inside the package headspace. According to Day (2008), active packaging is "packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system" (p. 1). By combining this definition with the explanation of others, active packaging can be described as packaging that actively interacts with the packaged foods and the surroundings inside the package, in a way that stabilizes the conditions inside the package in order to prolong food safety and quality (Han et al., 2014; Li et al., 2012; Remya et al., 2016).

If the antimicrobial packaging functions as intended, it can be an important factor to maintain the quality and safety of the packaged food over prolonged time compared to conventional packaging methods (Han et al., 2014; Kong, Chen, Xing, & Park, 2010; Remya et al., 2016; Torlak & Sert, 2013). For muscle foods, surface contamination is the major issue. The muscle is sterile prior to handling, and any contaminations occurring during handling will be present at the surface of the product. Muscle foods would therefore benefit with antimicrobial packaging materials that inhibit growth of bacteria and other microbiota on the surface of the product (Ouattara, Simard, Piette, Bégin, & Holley, 2000; Remya et al., 2016; Torlak & Sert, 2013). Application of the active agent inside the conventional package would be satisfying; at the same time that it would cause a need for an additional step in the packaging process. This disadvantage would be avoided if the active agent was incorporated into the packaging material. This way, the packaging process would be equal to the conventional method, and the concentration of the agent would be higher at the food surfaces that are in contact with the material. Therefore, if the packaging material would be used for vacuum-packaging, the risk associated with surface-contamination would be significantly reduced compared to conventional packaging (Ouattara et al., 2000). Among the active agents that can be incorporated into the packaging material is chitosan. Chitosan-containing films have several advantages compared to conventional packaging films, as chitosan has both antimicrobial properties and metal chelation abilities (Wickramarachchi et al., 2016).

2.4 Chitosan

During the last decade, chitosan has received a lot of attention due to its antimicrobial properties against fungi and bacteria (Al-Sagheer et al., 2014; Fernandez-Saiz, Lagaron, & Ocio, 2009; G.-H. Wang, 1992). These properties make chitosan well suited for use in active packaging, as it can possibly prolong the shelf life and quality of foods (Arancibia et al., 2014; Portes et al., 2009). Chitosan (Figure 2.1B) is made by deacetylation of chitin (Figure 2.1A) in an alkaline environment (Dutta et al., 2012; Rabea et al., 2003). Chitin is the second most common natural biopolymer after

cellulose, and it is found in large amounts in the shells of crustaceans (Al-Sagheer et al., 2014; No, Meyers, Prinyawiwatkul, & Xu, 2007; Rabea et al., 2003). It can also be produced by fungi (Devlieghere, Vermeulen, & Debevere, 2004; Portes et al., 2009), or found in insects and mushrooms (Alishahi & Aïder, 2012; García et al., 2010; Sébastien et al., 2006), which means that there are many natural sources for chitin that otherwise are not utilized.

Chitosan is a cationic polysaccharide consisting of two monosaccharides (Alishahi & Aïder, 2012; Bonilla et al., 2013). The degree of deacetylation varies from 75 % to 95 %, which gives chitosan with different molecular weights (Alishahi & Aïder, 2012). The reason why chitosan is being investigated with relation to food packaging, is that it has many features that are wanted in active packaging materials, such as biodegradability, biocompatibility and non-toxicity (Albertos et al., 2015; Dutta et al., 2012; Manni, Ghorbel-Bellaaj, Jellouli, Younes, & Nasri, 2010), at the same time that it holds antimicrobial properties (Albertos et al., 2015; Bonilla et al., 2013; Liu, Qin, He, & Song, 2009). However, the functional properties of chitosan vary depending on the molecule's characteristics, the general composition of the system, and environmental factors such as temperature (Zivanovic, Chi, & Draughon, 2005).

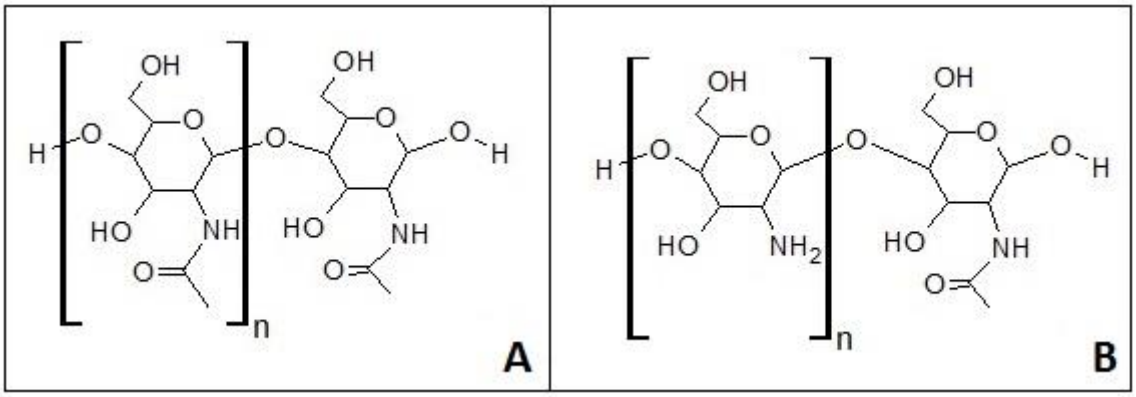

Figure 2.1: The chemical structure of chitin (A) and its deacetylated derivate chitosan (B) (Dutta et al., 2012).

2.4.1 Antimicrobial effect of chitosan

Arancibia, Alemán, López-Caballero, Gómez-Guillén, & Montero (2015) explain the antimicrobial effect of chitosan based on its structure. Chitin being a natural antimicrobial in the organism which it is purified from, strengthens the theory that chitosan can be used as an antimicrobial in multiple settings (Arancibia et al., 2015). The fact that chitosan has an antimicrobial effect is agreed upon among researchers, but whether or not there is a difference in the efficacy against Gram-positive and Gram-negative bacteria is widely discussed (Rhim et al., 2009; Torlak & Sert, 2013; Vardaka, Yehia, & Savvaidis, 2016).

Many research groups agree that the antimicrobial effect possibly is caused by an interaction between the positive charges on chitosan and the negative charges on the outside of the microbial cell membrane. Bonilla et al. (2013) explain that the interaction has a negative effect on the nutrient supply to the cell which leads to cell death. No et al. (2007) and Yilmaz Atay and Çelik (2017) on the other hand, say that the interaction causes a leakage in the cell membrane, leading to intracellular compounds leaking out of the cell. L. Wang et al. (2011) do not give an explanation of the exact mechanism, but agree that the aforementioned interaction caused by charges causes the microbial cell to die. Martínez-Camacho et al. (2010) have suggested three different mechanisms; (1) that the

negative charged chitosan interacts with the bacteria cell and cause death of the bacterium either by causing leakage of intracellular compounds, or by affecting the nutrient supply to the cell, (2) that chitosan functions as a chelating agent, limiting the availability of metals that are needed by the cell, or (3) that low molecular weight chitosan is able to enter the cell, upon which it disturbs the mRNA synthesis, affecting the production process of proteins and inhibiting enzymes.

The antibacterial effect of chitosan is affected by both intrinsic and extrinsic factors (Zivanovic et al., 2005). In addition, the physical state of chitosan is said to be of importance, as chitosan is assumed to be more efficient in a solution than in a film (Leceta, Guerrero, Ibarburu, Dueñas, & De La Caba, 2013; Zivanovic et al., 2005). This is because the chitosan molecules are more easily available when in a solution than they are as part of a rigid film (Abdollahi et al., 2012; G.-H. Wang, 1992).

2.4.2 Making active packaging films incorporated with chitosan

One of the difficulties regarding production of chitosan-containing films is the fact that the films cannot be made by heat-disintegration of the components. Chitosan has to be solved in acidic solutions, otherwise the film resulting will not be homogenous (Bonilla et al., 2013; No et al., 2007).

Films made of chitosan alone are rigid, brittle and unable to keep gasses and moisture inside a package (Leceta, Guerrero, & De La Caba, 2013). Therefore, the active packaging material must be made of a combination of chitosan and other natural polymer(s) in order to have a satisfying film. Among the sustainable polymers, polylactic acid (PLA) is among those most widely investigated and used (Turalija, Bischof, Budimir, & Gaan, 2016). It is produced from renewable resources such as starch from corn and rice (Bonilla et al., 2013), and similarly to chitosan, it is degradable and generally recognized as safe for use in contact with foods (Chariyachotilert, Joshi, Selke, & Auras, 2012; Darie et al., 2014; Sébastien et al., 2006). Neither PLA nor its degradation products are toxic or carcinogenic to humans. PLA films are transparent, making it very interesting for use as a replacement for the non-degradable plastics that are in use today (Turalija et al., 2016). PLA is a commonly tested compound in chitosan films to lower the sensibility to water vapor (Bonilla et al., 2013; Sébastien et al., 2006). The combination of chitosan and PLA gives non-toxic, biodegradable films with good mechanical properties, which are highly resistant to permeability by gasses and water (Râpă et al., 2016). It is considered one of the most promising materials because it offers a possibility for a natural film that is solid and transparent, and it can be made using the production technology that is in use today (Bonilla et al., 2013). Despite all the positive abilities of PLA, it is not easy to make a product that is able to compete against the well-known materials in use today, because the production costs for PLA films are higher than the costs for production of conventional films (Li et al., 2012).

When making a film, the varying chemical properties of chitosan must be taken into consideration, as they are likely to affect the quality of the film (Leceta, Guerrero, & De La Caba, 2013). Zivanovic et al. (2005) reported that high molecular weight of chitosan was an advantage in film-making, as the films containing chitosan with higher molecular weight were stronger compared to those made with lower molecular weight chitosan. This implies that during preparation and testing of chitosan for production of packaging films, one should compare molecular weight to antibacterial effect in order to have the best possible antimicrobial packaging film.

2.5 Foodborne bacteria

Foodborne bacteria are a continuous concern related to human health worldwide. The expectation is that active, antimicrobial packaging methods can reduce the risk by controlling the growth of microorganisms in foods (Torlak & Sert, 2013). The bacteria present in different foods vary depending on type of raw material, production method, handling hygiene, and processing (Hobbs, 1982). In the food, spoilage bacteria might induce reactions that provoke changes in flavor, odor, color or other sensory properties (Lucera et al., 2012). In addition to spoilage bacteria, there are pathogenic microorganisms that can cause illness to those consuming contaminated foods even if there are no sensory changes (Lucera et al., 2012). However, today there are methods that can be used to delay these processes, and hence prolong the shelf-life of foods. These methods can be alteration of intrinsic factors such as pH and water activity, or extrinsic factors such as storage time and temperature (Lucera et al., 2012). Every bacteria found in foods have their own nutritional requirements, and they are all predictably affected by the different parameters in the environment (Jay, Loessner, & Golden, 2005).

Two of the common pathogenic bacteria in foods are *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*) (Lucera et al., 2012; Trudeau, Vu, Shareck, & Lacroix, 2012; G.-H. Wang, 1992). In experiments, these bacteria are often used as a standard for Gram-negative and Gram-positive bacteria, respectively (Leceta, Guerrero, Ibarburu, et al., 2013; Remya et al., 2016). They were therefore selected as test organisms for the experiments. Both bacteria can cause serious illness, and food contaminated with *E. coli* and *S. aureus* represents a risk to human health (Zhang et al., 2016).

2.5.1 *Staphylococcus aureus*

S. aureus is a facultative anaerobe, Gram-positive cocci that can cause a range of illnesses in humans (Baptista et al., 2015; Shi et al., 2016; Wickramarachchi et al., 2016). It is a foodborne pathogen, and it is considered important with relation to public health risks (Nan, Yang, & Ren, 2015; Trudeau et al., 2012). It is a bacterium that can grow in many different environments, as it tolerates a wide range of both temperature, salt concentration, and pH (Baptista et al., 2015; Wickramarachchi et al., 2016). It grows at temperatures from 7-48°C and at pH 4.0-9.8. Its optimum temperature for growth is 30-37°C and optimum pH is 6.0-7.0. Most strains of the bacterium tolerate salt concentrations at 10%, but some species can grow at salt concentrations up to 20% (Jay et al., 2005).

In experiments, *S. aureus* is used as standard for Gram-positive bacteria, but it is also a good indicator for hygiene in food productions where human handling is involved (Hobbs, 1982). As 20-30% of humans are constantly carrying the bacterium on their skin and in their mucosal membranes, there is a major risk for contamination with the bacterium from hands that handle food products if they do not wear gloves (Fetsch et al., 2014). It is a common foodborne pathogen, and as it produces heat resistant enterotoxins in the food, it can cause illness at consumption of the foods even if the bacteria itself does not tolerate the heat treatment (Fetsch et al., 2014; Shi et al., 2016; Trudeau et al., 2012). In the European Union, staphylococcal toxins are the major cause of food-related illness (Fetsch et al., 2014). Food poisoning by staphylococci is recognized by acute symptoms including pain in the stomach, diarrhea, and vomiting (Fetsch et al., 2014).Development of methods that can potentially limit or suppress general growth and production of toxins by *S. aureus* in foods is of great interest as it is very common and potentially dangerous for consumers (Shi et al., 2016).

2.5.2 *Escherichia coli*

E. coli is a rod shaped, facultative anaerobe, Gram-negative bacterium that belongs to the *Enterobacteriaceae* family (Baker, Rubinelli, Park, Carbonero, & Ricke, 2016; Wickramarachchi et al., 2016). It is part of the normal flora in the intestinal tract of warm-blooded animals, and can therefore be used as an indicator organism for fecal contamination of both foods and water (Jay et al., 2005). The bacterium is common in the intestine, and one can thereby understand that it is able to survive a pH≈2 because it must pass through the acidic environment in the stomach to reach the intestine, but its ideal pH for growth is in the range of 5-9 (Baker et al., 2016; Lee, Kim, & Kang, 2015). It grows at temperatures from 7-48°C with an optimum temperature at 37°C (Jay et al., 2005). This as well confirms the fact that it is dangerous for humans, as the body temperature is its optimum growth temperature, at the same time that it survives most pH levels.

Although *E. coli* is a part of the normal intestinal microbiota in humans and other mammals, some strains are potentially pathogenic in humans (Baker et al., 2016; Pariz Maluta et al., 2014). These strains might cause different illnesses among which serious foodborne diseases are common (Wickramarachchi et al., 2016). Like *S. aureus* it does not form spores, which means that it does not survive the highest temperatures, and as its toxins are produced inside the consumer, it is enough to inactivate the vegetative cells in the food to avoid illness (Jay et al., 2005).

2.6 Test methods

In order to find a good, reliable method to detect an antimicrobial effect of chitosan, different methods were tried (Figure 2.2). First, a contact test was performed by which 13 different chitosan-containing films were tested by an ISO-method. The films had different combinations of components, in order to see if the effect of chitosan was affected by the other components of the film. The films also had different concentrations of chitosan, to test whether or not there was a noticeable difference in the activity depending on the chitosan concentration.

As the ISO-method had a lot of uncertainties, a method that was thought to give more stable results was used. In this method, different amounts of chitosan film or powder were added to a liquid bacteria culture with a known bacterial concentration. The solutions were incubated with regular sampling during the incubation time. The film was tested first, and as the results seemed promising, the same method was used to test corresponding amounts of chitosan powder.

Next, chitosan powder was dissolved in acetic acid to make chitosan colloid. Chitosan colloid means that chitosan powder is dissolved in a liquid so that the two substances cannot be separated by filtration or other separation methods. This variant of chitosan was tested by use of a growth analyzer, yielding growth curves. These curves were then used to compare bacteria solutions with and without chitosan in order to see if there seemed to be an antibacterial effect of chitosan.

The liquid tests and the testing with chitosan colloid gave promising results regarding antimicrobial activity, leading to testing of a chitosan-containing packaging material on food. Since neither chitosan powder nor colloid can be used for food packaging, chitosan trays made from chitosan film were used in this experiment. Smoked salmon was packaged in trays, and sampling was performed during storage.

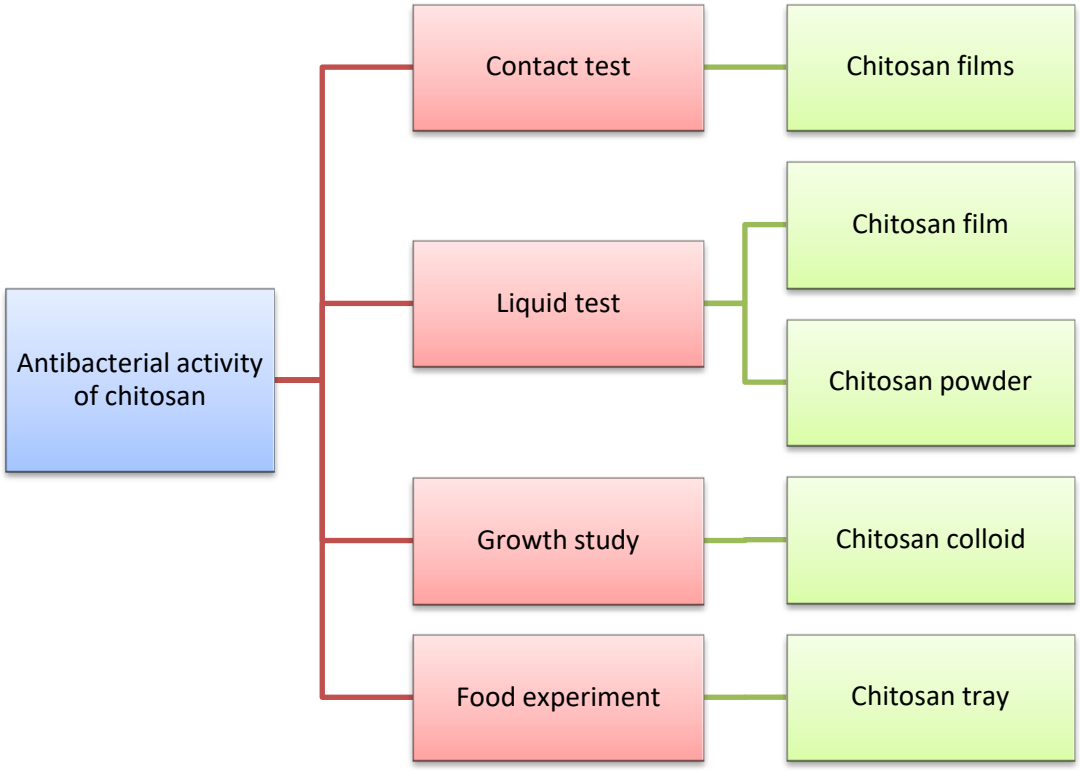

Figure 2.2: Overview of the methods used to test the antibacterial activity of chitosan

3 Materials and methods

3.1 Bacteria strains

The bacteria used in the experiments were either from the Culture collection from the University of Gothenburg (CCUG) or from the American type culture collection (ATCC). The Gram-positive *S. aureus* CCUG 1828 and the Gram-negative *E. coli* CCUG 10979 were used in most experiments. In the experiments with the ISO-method, *S. aureus* ATCC 6538 and *E. coli* ATCC 8739 were used.

The bacteria were stored in Microbank^{TM} tubes (Microbank, Pro-lab Diagnostics, Canada) at -80°C. The Microbank tubes are pre-sterilized 2 ml vials containing approximately 25 colored beads in a preservative liquid. Colonies were transferred from plates with the bacteria into Microbank tubes by use of a sterile loop. From the producer, the beads had been treated chemically in order to improve the bacterial adhesion, making the bacteria stick to the beads. In order to ensure adhesion to all beads, the tube was inverted three to four times. The preservative liquid ensures survival of bacteria, and also ensures higher quantitative recovery after storage ("MicrobankTM Worldwide Performance Portfolio," 2011). Before use in experiments, beads were transferred to the broth or agar plates given in the methods, and incubated at the given temperature and time.

3.2 Chitosan films

The various chitosan films and tray used in the project were produced by ICPAO S.A. (Medias, Romania). The films consisted of different components (Table 3.1) in order to test if there was a difference in the antimicrobial activity depending on the film composition.

The chitosan (CS) used in the experiments was distributed by Sigma-Aldrich (Saint Louis, USA, batch: STBF8484V). It was medium-molecular weight with a degree of acetylation at 83.2% and a viscosity of 328CPS when 1% chitosan is dissolved in 1% acetic acid. Two modified variants of the chitosan was also used; one that had been modified by encapsulating rosehip oil (CS-M), and one which had been further modified by addition of purified sodium montmorillonite (Cloisite C30B) (CS+C30B). Both additives are known to have antimicrobial properties.

All films were PLA-films, with PLA as the major component. Generally, PLA films are transparent and hold many of the favorable characteristics that chitosan has. All films also contained 1% vitamin E as a bioactive agent.

Two of the test films contained tributyl o-acetyl citrate (ATBC), which is used as an eco-friendly biodegradable plasticizer to reduce the PLA brittleness (Râpă et al., 2016). The ATBC used had a molecular weight of 406 g/mole, a density of 1.055g/cm^3 at 25°C, and a purity of 99.35%. The ratio between PLA and ATBC was 4:1 in the films.

Another plasticizer, polyethylene glycol BioULTRA 4,000 (PEG) (Sigma-Aldrich), was used in some films. It is added to link the polymer to the active agent, so that the active agent can move enough to be able to be in contact with the microorganisms on the surface of the foods (Appendini & Hotchkiss, 2002). The PEG used had a molecular weight of 4,016 g/mole and a melting point of 61°C. A component that was used in many of the films is Masterbatch Lapol 108 (MB) (LAPOL LLC, Santa Barbara, USA). MB is a patented bioplasticizer that is used to increase the PLA tenacity, flexibility, and melt strength. Dellite HPS (HPS) is unmodified nanoclay based on montmorillonite. It was added

to improve the hydrophilicity, which should enhance the antibacterial effect of the film (Darie et al., 2014). BYK-P 4101 (BYK) is a copolymer that was added to improve the conditions for processing and maintaining or improving the physical and mechanical properties such as tensile strength and modulus of elasticity. It is approved for applications that involve contact with food.

The films were prepared by melt blending at a temperature of 170±5°C, with mixing for 6 minutes at 60 rpm. PLA and MB were dried at 50°C for 24h before use to reduce the moisture content, and the modified chitosan was dried at 40°C for 4h. The resulting mixtures were hot-pressed at 175°C for a total of 15min with a pressure of 147 bars, followed by cooling for 20min. This resulted in films with 200*200*0.1 mm dimensions.

Table 3.1: List of the chitosan-containing films used in the project.

Film composition	**% chitosan**	**% PLA**	**Plasticizer**		**Used in experiment**		
			ATBC	MB	Contact test	Liquid solution	Food experiment
PLA/ATBC/CS 3%	3	77.6	X	-	X		
PLA/ATBC/CS 5%	5	76.0	X	-		X	
PLA/MB/PEG 8%	0	79.2	-	X	X		
PLA/MB/PEG 8%/CS-M 1%	1	78.4	-	X	X		X
PLA/MB/PEG 8%/CS+C30B 1%	1	78.4	-	X	X		
PLA/MB/PEG 8%/CS+C30B 2%	2	77.6	-	X	X		
PLA/MB/PEG 8%/CS+C30B 3%	3	76.8	-	X	X		
PLA/MB/PEG 6%/CS-M 1%	1	78.4	-	X	X		
PLA/MB/PEG 6%/CS+C30B 1%	1	78.0	-	X	X		
PLA/MB/CS-M 1%	1	78.4	-	X	X		
PLA/MB/CS-M 1%/BYK 1%	1	77.6	-	X	X		
PLA/MB/CS-M 1%/BYK 3%	1	76.0	-	X	X		
PLA/MB/CS 1%/HPS 3%	1	76.0	-	X	X		
PLA/MB/CS1%/HPS3%/BYK3%	1	73.6	-	X	X		

3.3 Antibacterial activity on plastic surfaces

The International Organization for Standardization (ISO) is an organization that set standards for many different industries. Its standards ensure that experiments are conducted in the same way, and that the results are reliable (Koppell, 2011). Their method ISO-22196 “Plastics – measurement of antibacterial activity on plastics surfaces” (2007) was used to test the antimicrobial effect of chitosan films. In this method, a defined volume of bacteria solution was placed on a plastic surface that was to be tested. On top of the bacterial solution, a neutral cover sheet of a defined size was placed so that the contact surface between the bacteria and the test material was known. By being consequent on the sizes and volume used, it was possible to compare results between parallels and experiments as the conditions were equal for all samples.

3.3.1 Bacteria strains

S. aureus CCUG 1828 and *E. coli* CCUG 10979 were used for testing of the antimicrobial effect of the chitosan-containing film PLA/ATBC/CS3. *S. aureus* ATCC 6538 and *E. coli* ATCC 8739 were used to test the other films.

3.3.2 Preparation of culture media

Nutrient broth

Nutrient broth (NB) was prepared by dissolving 3.0 g meat extract (Merck, Darmstadt, Germany), 10.0 g peptone (Sigma-Aldrich, Saint Louis, USA) and 5.0 g sodium chloride (Merck, Darmstadt, Germany) in 1000 ml distilled water. The NB was then diluted 1:500 with distilled water, and pH adjusted to 7.1±0.1 by use of HCl or NaOH prior to sterilization by autoclaving at 121°C for 15 min. The broth was allowed to cool down on the bench before storage in a cool room at 4°C for a maximum of 30 days.

Soybean casein digest broth with lecithin and polyoxyethylene sorbitan monooleate (SCDLP)

Soybean casein digest broth with lecithin and polyoxyethylene sorbitan monooleate (SCDLP) was prepared by dissolving 30.0 tryptone soya broth (TSB) (Oxoid, Basingstoke, Hants, UK) and 1.0 g lecithin (AppliChem, Darmstadt, Germany) in 1000 ml distilled water, before addition of 7.0 ml Tween 80 (Sigma-Aldrich, Saint Louis, USA). The pH was adjusted to be in the range of 7.1±0.1 by use of HCl or NaOH prior to sterilization by autoclaving at 121°C for 15 min. The medium was allowed to cool down on the bench before storage in a cool room at 4°C for a maximum of 30 days.

Phosphate buffer solution

Phosphate buffer solution was prepared by dissolving 34.0 g potassium dihydrogen phosphate (Sigma-Aldrich, Steinheim, Germany) in 500 ml distilled water before adjustment of the pH to 7.1±0.1 by use of HCl or NaOH. When the pH was within the desired range, distilled water was added until a final volume of 1000 ml PBS. The solution was sterilized by autoclaving at 121°C for 15 min. The solution was allowed to cool down on the bench before storage in a cool room at 4°C for a maximum of 30 days.

Phosphate-buffered physiological saline

Physiological saline was prepared by dissolving 8.5 g sodium chloride (Merck, Darmstadt, Germany) in 1000 ml distilled water. The solution was sterilized by autoclaving at 121°C for 15 min. The solution was cooled on the bench before storage in a cold room at 4°C for a maximum of 30 days. The phosphate buffer solution was then diluted in the physiological saline to an 800-fold volume. This solution as well was stored in a cold room at 4°C for a maximum of 30 days.

Plate count agar

Plate count agar (PCA) was prepared by dissolving 22.5 g Plate count agar (Merck, Darmstadt, Germany) in 1000 ml distilled water on a stirrer. The pH was adjusted to 7.1±0.1 by use of HCl or NaOH prior to sterilization by autoclaving at 121°C for 15 min. When the agar was used to make plates, approximately 15 ml agar was poured into sterile petri dishes with a diameter of 9cm and allowed to solidify before storage in a cold room at 4°C for a maximum of 30 days. If the agar was to be used for pour plates, the autoclaved bottle was allowed to cool on the bench before storage in a cold room at 4°C for a maximum of 30 days. Before use, the agar was heated by boiling to melt the agar, and allowed to cool until a stable temperature of 45 °C before application into plates with bacteria.

3.3.3 Procedure

The bacteria used in the test were prepared by making overnight-cultures (ON-cultures) by plating of a bead from Microbank tube onto PCA. One plate was prepared for each bacterium. The plates were

incubated at 37°C for 24h. Test inoculum was prepared by transferring a loop of the ON-culture to 5ml 1/500 NB and mixing until the bacteria were evenly dispersed in the solution. An enumeration of the test inoculum was required prior to testing of the materials, in order to ensure that the start concentration was correct so that the test could be valid according to the ISO standard.

The enumeration was performed by use of a Thoma cell counting chamber (Celeromics Technologies, Cambridge, UK). The chamber is divided into 16 large squares (0.2mm×0.2mm), which are again divided into 25 small squares (0.05mm×0.05mm). For the enumeration, the chamber was filled with bacteria solution and read by use of a microscope with 400x magnification. The average count of at least 5 large squares multiplied by $2.5*10^5$ gave the bacterial concentration as colony forming units per ml (cfu/ml). If there was a high concentration of bacteria, small squares could be counted and the average count was then multiplied with $4*10^6$ to find the bacterial concentration as cfu/ml. The desired start concentration was $6x10^5$ cfu/ml. If the enumeration resulted in a higher concentration, the solution was diluted in 1/500 NB. If the enumeration resulted in a lower concentration, another loop of bacteria was added from the ON-culture, and the solution was enumerated and diluted as necessary. The bacteria solution was kept on ice for a maximum of 2h before use.

The method required use of both untreated and treated test material. Stomacher bags were used as untreated material and chitosan films as treated test material. The test material size was 50mm×50mm, and the cover films which were made of stomacher bag had a size of 40mm×40mm. The materials were placed on a sterile bench and cut with a scissor that had been sterilized by use of ethanol. In accordance with the standard, the test film was put in the bottom of an empty, sterile petri dish, followed by application of 400 µl test inoculum before placing the cover film (Figure 3.1). The cover film was carefully placed by use of sterile tweezers in order to maintain the sterility of the film, but also to ensure that the test inoculum did not leak over the edge of the treated test material. Three parallels of each treated test material were used, and six parallels with untreated test material were included as control.

Half of the dishes with untreated test material were recovered straight after inoculation, while the rest of the dishes were incubated at 37°C for 24±1 h in a humidity chamber with a humidity of at least 90%. The recovery was performed by washing with 10ml SCDLP, ensuring a total wash by pipetting up and down a couple times with a sterile pipette. The SCDLP from the dishes was transferred from the dish into sterile, labelled tubes before further processing. When all dishes had been washed and the SCDLP collected, the samples were enumerated by making a 10-fold dilution series in 1/800 phosphate-buffered physiological saline. The dilutions were plated in duplicates by making pour plates with PCA. 1ml of the dilution was placed in a sterile petri dish before melted PCA was poured on, and the two solutions mixed thoroughly. When the plates had solidified, they were incubated at 37°C for 40-48h. Plates containing 30-300 colonies were used for calculations where such plates existed, otherwise the bacterial number from the least diluted plate was used.

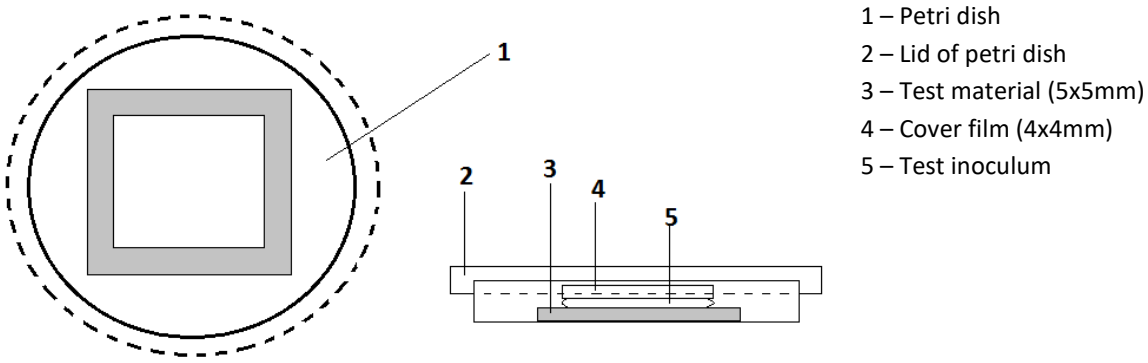

Figure 3.1: Demonstration of placement of films and test inoculum in petri dish.

3.3.4 Calculations

Calculation of cells/cm²

The number of bacteria recovered from the dishes was calculated by the following equation:

$$N = (100 * C * D * V)/A$$

Where:

N=recovered bacterial number in cells/cm^2;

C=average plate count from the duplicates;

D=dilution factor for the plates enumerated;

V=volume of SCDLP, in ml;

A=surface area of cover film, in mm^2.

Conditions for a valid test

There were three conditions that should be met in order to have a valid test:

1: $L_{max} - L_{min}/L_{mean} \leq 0.2$
where L_{max} is the base 10 logarithm of the highest number of bacteria recovered on a specimen after incubation, L_{min} is the base 10 logarithm of the lowest number of bacteria recovered on a specimen after incubation, and L_{mean} is the base 10 logarithm of the mean number of bacteria recovered on a specimen after incubation.

2: The samples with untreated test materials recovered immediately after inoculation should have an average bacterial number between 6.2x10^3 and 2.5x10^4 cells/cm^2.

3: The number of bacteria recovered from the samples with untreated test material after incubation for 24h should not be lower than 6.2x10^1 cells/cm^2.

If one or more of these conditions were not met, the test was not valid.

Calculation of log reduction

In order to compare the results from the different films, the log reduction of bacteria was calculated by this equation:

$$log\ reduction = \log(A) - \log(B)$$

where:

A=initial count of bacteria in cells/cm^2 in the control sample;
B=bacterial count in cells/cm^2 after a defined incubation time with an antimicrobial film.

3.4 Test of chitosan in liquid solution

An alternative method to ISO 22196 used for testing the antimicrobial properties of chitosan was modified from Rhim, Hong, Park and Ng (2006). Rhim et al. used 100cm^2 of the test film in 100ml bacteria solution, with sampling after 0-2-4-6-8h of incubation. The method was modified so that bacteria were grown in broths together with various concentrations of chitosan, either film or powder. Sampling was performed during incubation, and the bacterial numbers at each sampling time was plotted into a graph to see the difference between the different concentrations of chitosan. This is a quantitative method used to study the antimicrobial activity of increasing concentrations of chitosan in liquid bacteria solutions.

3.4.1 Bacteria strains

The bacteria used for the tests were *S. aureus* CCUG 1828 and *E. coli* CCUG 10979.

3.4.2 Preparation of culture media

Tryptone soya broth

Tryptone soya broth (TSB) was prepared by dissolving 15g TSB in 500 ml distilled water on a stirrer before adjustment of the pH to 7.1±0.1 by use of HCl. The broth was sterilized by autoclaving at 121°C for 15 min. The broth was cooled on the bench before storage in a cold room at 4°C for a maximum of 30days.

Tryptone soya agar

Tryptone soya agar (TSA) was prepared by dissolving 20g tryptone soya agar (Oxoid, Basingstoke, Hants, UK) in 500 ml distilled water on a stirrer before adjustment of the pH to 7.1±0.1 by use of HCl. The agar was sterilized by autoclaving at 121°C for 15 min. It was then cooled on the bench before storage in a cold room at 4°C for a maximum of 30days. Prior to use for pour plates, the agar was melted either by boiling or by autoclaving, and allowed to cool until a stable temperature of 45°C before application into plates with bacteria.

Brain-heart infusion broth

Brain-heart infusion broth (BHI) was prepared by dissolving 18.5g brain-heart infusion broth (Merck, Darmstadt, Germany) in 500 ml distilled water on a stirrer before adjustment of the pH to 7.1±0.1 by use of HCl. The solution was sterilized by autoclaving at 121°C for 15 min. The solution was cooled on the bench before storage in a cold room at 4°C for a maximum of 30days.

Brain-heart agar

Brain-heart agar (BHA) was prepared by dissolving 26g brain-heart agar (Merck, Darmstadt, Germany) in 500 ml distilled water on a stirrer before adjustment of the pH to 7.1±0.1 by use of HCl. The solution was sterilized by autoclaving at 121°C for 15 min. The solution was cooled on the bench before storage in a cold room at 4°C for a maximum of 30days. Prior to use for pour plates, the agar was melted either by boiling or by autoclaving, and allowed to cool until a stable temperature of 45°C before application into plates with bacteria.

Peptone water

0.1% (w/v) peptone water was prepared by dissolving 0.5 g peptone water (Oxoid, Basingstoke, Hants, UK) in 500 ml distilled water on a stirrer before adjustment of the pH to 7.1±0.1 by use of HCl or NaOH. The solution was sterilized by autoclaving at 121°C for 15 min. It was cooled at the bench before storage in a cold room at 4°C for a maximum of 30days.

3.4.3 Procedure

First, an ON-culture was made for each bacterium. *E. coli* was grown in 30ml TSB at 30°C, while *S. aureus* was grown in 30ml BHI at 37°C for 17±2 h. After incubation, the cell cultures were centrifuged for 5min at 7000*g* and 4°C, and the supernatant was discarded before washing the pellet with 0.1% peptone water, followed by another centrifugation for 5min at 7000*g* and 4°C. The supernatant was discarded, and the pellet solved in 100ml TSB or BHI, corresponding to which broth had been used previously. This gave a bacterial concentration of log 8 cfu/ml. Depending on the experiment this solution was further diluted in sterilized distilled water. The bacteria solution was transferred to sterile tubes, 25ml to each, before addition of chitosan film or powder. Test tubes without chitosan were included for each bacterium as a control.

The chitosan-containing film used in these experiments was PLA/ATBC/CS5. 0, 10 or 25cm^2 of the film was added in the tubes with bacteria solution (Figure 3.2). For the chitosan powder, 0, 8 and 20mg was used (Figure 3.3), as these amounts were equivalent to the amounts of film tested.

The tubes were incubated horizontally on an orbital shaker at 70rpm. As the incubation time and temperature was different between the experiments, the incubation time and temperature are given for each experiment in the result section. At sampling, 10-fold dilution series were made in 0.1% peptone water before making pour plates with BHA or TSA, equivalent to the previously used broth. The BHA dishes were incubated at 37°C and the TSA dishes at 30°C for 40-48h before enumeration.

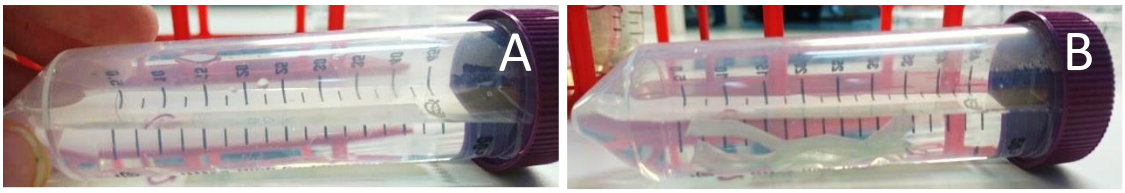

Figure 3.2: Test tubes from testing of chitosan-containing film (PLA/ATBC/CS5) in a bacteria solution with a start concentration of *E. coli* about log 5 cfu/ml. A=no film, B=25cm^2 film.

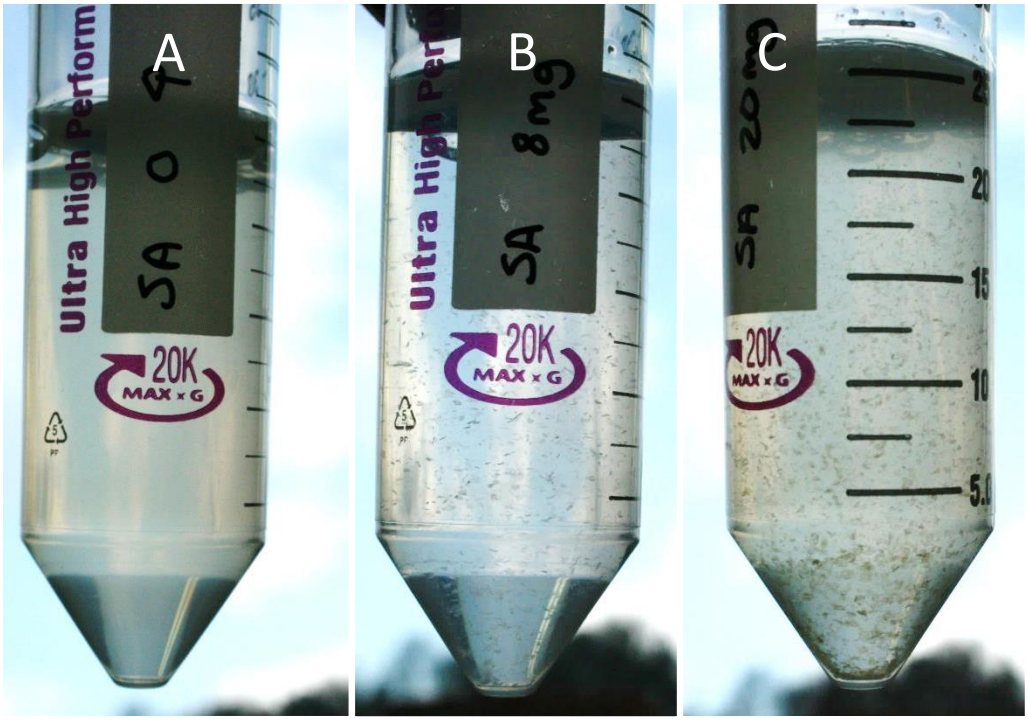

Figure 3.3: Test tubes from testing of chitosan powder in bacteria solution with a start concentration of *S. aureus* about log 7 cfu/ml. A=0 mg, B=8 mg, C= 20 mg chitosan powder.

3.5 Growth curve analyses

As the results from testing of chitosan-containing film and chitosan powder in liquid solution were promising, chitosan powder was dissolved to see if its effect would be better when dissolved, as it then will be more easily available for the bacteria (Kong et al., 2010; Leceta, Guerrero, Ibarburu, et al., 2013; Zivanovic et al., 2005). Medium molecular weight chitosan is only soluble in organic acids with a pH below 6.3 (Massouda et al., 2012), and as it was given in the Certificate of Analysis from the producer that it is soluble in 1% acetic acid, this was chosen as solvent. However, the acid tolerance of the two test bacteria had to be taken into consideration, as they do not grow at a too low pH. The effect of the chitosan colloid was tested using the BioScreen C Microbiology Reader (Bioscreen) (Oy Growth Curves Ab Ltd., Turku, Finland).

3.5.1 Bacteria strains

The bacteria used for the test were *S. aureus* CCUG 1828 and *E. coli* CCUG 10979.

3.5.2 Preparation of culture media

Acetic acid

Various concentrations of acetic acid used to dissolve chitosan powder were prepared by dilution of 100% glacial acetic acid (Merck, Darmstadt, Germany) in distilled water. The acid was sterilized by filter sterilization before use in experiments.

Chitosan colloid

The chitosan colloid was prepared by dissolving 0.050g chitosan powder in 10ml acetic acid to have a concentration of 0.5% chitosan. In order to avoid contamination from the equipment, the bottle and magnet was sterilized by autoclaving at 121°C for 15 min before use. Chitosan was weighed and added to the bottle before addition of acetic acid of the desired concentration. The solution was left stirring overnight to dissolve the chitosan powder.

3.5.3 Procedure

The bacteria solutions used in the experiment were prepared by first making an ON-culture for each bacterium. *E. coli* was grown in 30ml TSB at 30°C, while *S. aureus* was grown in 30ml BHI at 37°C for 17±2 h. After incubation, the cell cultures were centrifuged for 5min at 7000*g* and 4°C, and the supernatant was discarded before washing the pellet with 0.1% peptone water, followed by another centrifugation for 5min at 7000*g* and 4°C. The supernatant was discarded, and the pellet solved in 100ml TSB or BHI, respectively, to have a bacterial concentration of approximately log 8 cfu /ml. These solutions were further diluted in the respective broths to have the wanted bacterial concentration to run in Bioscreen.

Bioscreen reads optical density (OD) at given wavelengths in solutions that are placed in a micro-well tray inside the machine. The machine has room for two trays at a time, making it possible to run 200 samples at the same time. The conditions used for the experiments are given in Table 3.2 below.

Table 3.2: Conditions for Bioscreen used in the experiments

Parameter	**Value**
Incubation temperature	37°C
Read wave length	600nm
Time between measurements	10min
Shaking before measurements	10sec

A maximum of 200µl bacteria solution can be added to each well in the tray prior to running the machine, as larger volumes might cause mixing of samples during shaking. When testing the chitosan colloid, 160µl broth or bacteria solution and 40µl additive was added to each well. Three types of additives were used for each bacterial concentration; sterile water, acetic acid of the same concentration as in the chitosan colloid, and chitosan colloid. Samples with added water were included in the experiments in order to have a standard curve to compare the chitosan- and acid-containing samples with. Water was added so that the bacterial concentration would be the same in these samples as in the samples where acid or chitosan colloid was tested, as the bacteria solution is diluted when these solutions are added. The sample with addition of acetic acid was included in order to document that any antibacterial effect was caused by the chitosan, and not by the acid in which it had been dissolved. As a negative control, broth samples were included that was treated in

the same way as the bacteria-samples. This is important in order to detect any possible contamination of the different additives or the broth in which the bacteria solution has been diluted. Three parallels were included for each sample type in each run.

3.6 Experiments with food matrix

3.6.1 Preparation of culture media

Dilution liquid

Dilution liquid was prepared by dissolving 8.5g NaCl and 1g bacto peptone in 1000ml distilled water. The pH was adjusted to be in the range of 7.0±0.2, before the solution was autoclaved at 121°C for 15min. The liquid was cooled down before use, and stored in a cold room at 4°C for a maximum of 30days.

Long & Hammer agar plates

The Long & Hammer agar used for spread plates was prepared in accordance with Nordic committee on Food Analysis (NMKL) no. 184 (Nielsen, 2006).

Iron agar with L-cysteine

Iron agar was prepared by dissolving 43.6g Iron agar (Oxoid, Basingstoke, Hampshire, U.K.) in 1000ml distilled water. The pH was adjusted to be in the range of 7.4±0.2 by use of HCl or NaOH before autoclaving at 121°C for 15min. The agar was kept in bottles until use, as it was used to make pour plates. If the agar should be used straight away, it was cooled to 45°C before addition of L-cysteine. If not, it was kept in a cold room at 4°C for a maximum of 30days. It was then melted by boiling before use, and cooled down to 45°C before addition of L-cysteine.
L-cysteine was made by dissolving 5.0g L-cysteine in 100ml distilled water. 8ml/l L-cysteine solution was added to the cooled iron agar bottle before making pour plates.

When the pour plates had solidified, an overlay of Iron agar was added. This agar allows enumeration of H_2S-producing bacteria as black colonies, and total viable count as a total of black and white colonies.

3.6.2 Procedure

As the background for testing of the chitosan materials was to see if it could be used to inhibit foodborne bacteria, experiments were conducted in which a chitosan tray that had been produced for the project (Figure 3.4) was tested on smoked salmon. The chitosan tray consisted of PLA and chitosan with a chitosan concentration of 1% (for full composition, see table 3.1 page 10). A neutral plastic tray (HDPE) was used as control. The chitosan trays were placed inside trays that were identical to the control trays in order to make the packaging environment for all samples equal. The smoked salmon used was delivered from Lofotprodukt AS (Leknes, Norway), and all the fish fillets used were from the same production batch. This was important so that all the samples were as similar as possible, regarding both smoke time, storage time before start of the experiment, and bacteria in the samples.

Figure 3.4: Chitosan tray used in the experiment.

The fish fillets had been degutted and had the skin removed at the production location. At arrival, the fillets were cut in pieces equivalent to the size of the bottom of the trays that were to be tested (Figure 3.5A). The weight of the samples were 60-130g, as the thickness of the fillets vary depending on whether it is located near the tail or the head of the fish. The fish samples were put in the trays before they were flushed with 100% nitrogen gas and the packages were sealed (Figure 3.5B). At time of packaging, the fillets were 5 days old. For the first 27 days the samples were stored at 4°C, before the temperature was increased to 8°C at days 27-41 to provoke differences. Sampling was performed before storage, and then weekly from day 14 and throughout the storage period. This gave a total of five sampling times.

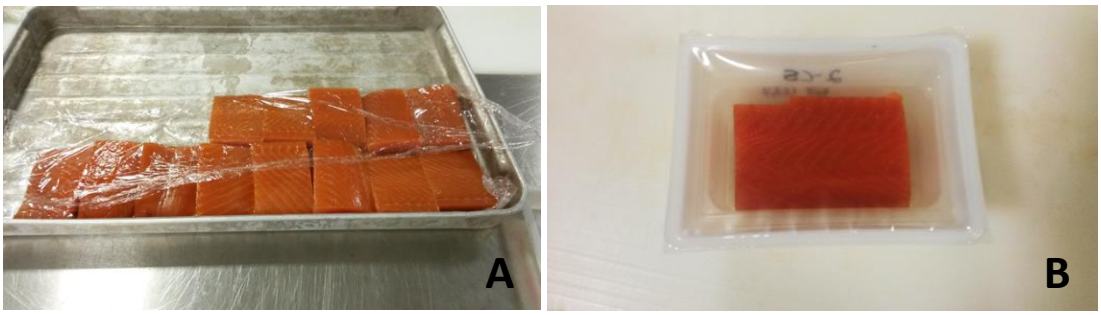

Figure 3.5: A – Fish pieces ready for packaging. B – Finished package of smoked salmon in a chitosan tray inside the HDPE tray.

At each sampling time, three parallels of each packaging material were used. The packages were opened by use of a sterile scalpel, and 20-25g from the bottom of the fish fillet, which had been in contact with the chitosan tray, was weighed in stomacher bags with filter. After weighing, the samples was diluted 10x (w/w) by use of dilution liquid. Then the sample was homogenized in a Stomacher 400 Laboratory Blender (Seward Medical, London, U.K.) for 120sec, before it was diluted as necessary prior to making spread-plates from 100µl sample on L&H-agar, and also plating by use of pour plate method with 1ml of the sample in Iron agar. The L&H-agar plates were incubated at 15°C for 5-7 days, and the iron agar plates were incubated at 20°C for 3 days before enumeration. The L&H plates allow enumeration of psychrotrophic bacteria, and the iron agar plates allow enumeration of H_2S producing spoilage bacteria.

4 Results and discussion

4.1 Antibacterial activity on plastic surfaces

The antibacterial films used in the experiments were produced by a research group (ICPAO) in Romania as a part of an EEC project. The films were still under development and an aim was to upscale production if it was possible to document sufficient results. Therefore, both practical applications in a food matrix and more specific antibacterial activity were of interest. The films used in the experiments were prepared by a melt blending technique to mix PLA with the other components in the films. The appearance of some films showed that air bubbles were trapped inside the film structure, and for some of the films chitosan was seen as black or brown particles embedded in the film (Figure 4.1). The air pockets give films that have an uneven surface, with areas that might have other physical properties than the rest of the film. In order to dissolve chitosan evenly in the film, chitosan has to first be solved in an acidic media (Bonilla et al., 2013; No et al., 2007). This would reduce the pH and influence the film properties, and was not used for these films. Instead, chitosan particles were unevenly spread in the film structure. The uneven distribution of chitosan resulted in films that had antibacterial properties only at specific points, compared to an optimal even distribution with antibacterial properties on the entire film surface. Chitosan is not volatile, and hence it only affects the bacteria that are in direct contact with the chitosan particles.

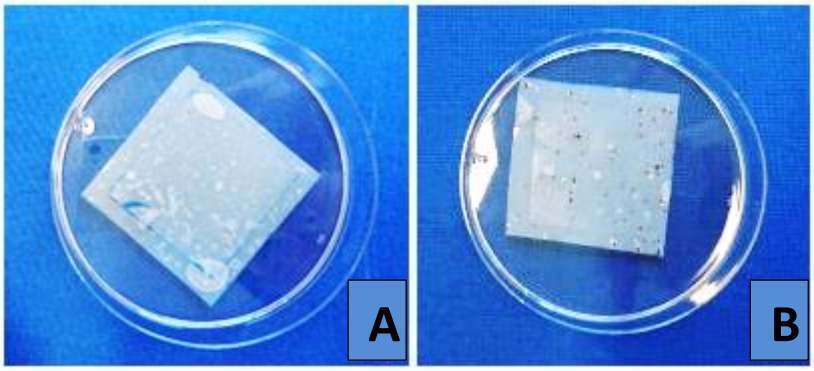

Figure 4.1: A – The film containing PLA/MB/CS-M1% had air bubbles trapped inside the film. B – The film containing PLA/MB/CS1%/HPS3%/BYK3% as an example of films that had the chitosan unevenly distributed throughout the film.

The first method tested was ISO 22196 “Plastics – Measurements of antibacterial activity on plastic surfaces”. This method is designed to measure the antibacterial properties of plastic materials. ISO methods have been tested and approved to give reliable and accurate results in accordance to their use. Torlak and Sert (2013) reported good results from their tests with this method, and they reported good results from use of this method on chitosan-containing films.

Several chitosan-containing films were tested by the ISO method, and the results showed great variation in the antibacterial activity (Table 4.1). None of the films had the same composition, and it is therefore likely that the differences detected were caused by the different components. For all tests using this method, values for bacterial numbers and standard deviations are shown in appendix A-1.

Table 4.1: Results from testing of chitosan-containing films by ISO-method 22196. The components in the films are explained in chapter 3.2 Chitosan films.

E. coli

Film	Log reduction
PLA/ATBC/CS3	1.54[a]
PLA/MB/PEG 8%/CS+C30B 1%	3.06
PLA/MB/PEG 8%/CS+C30B 2%	3.67
PLA/MB/PEG 8%/CS+C30B 3%	3.07
PLA/MB/CS-M 1%/BYK 1%	2.26
PLA/MB/CS-M 1%/BYK 3%	2.58

S. aureus

Film	Log reduction
PLA/ATBC/CS3	3.25[a]
PLA/MB/PEG 8%	4.38[b]
PLA/MB/PEG 8%/CS-M 1%	4.48[b]
PLA/MB/PEG 6%/CS-M 1%	4.48[b]
PLA/MB/PEG 6%/CS+C30B 1%	4.31[b]
PLA/MB/CS-M 1%	3.30[b]
PLA/MB/CS 1%/HPS 3%	1.73[b]
PLA/MB/CS 1%/HPS 3%/BYK 3%	2.98[b]

a: The represented value is a mean of two different experiments, each performed with three parallels.
b: According to the ISO-method used, the test result was invalid as the growth on the control sample with stomacher bag decreased too much during the 24h incubation between films.

For tests with *E. coli*, the best antibacterial effect was obtained with the film containing 8% of the plasticizer PEG and 2% chitosan with Cloisite 30B, which had a log reduction of 3.67 compared to the start concentration of the untreated sample. There was a clear antibacterial effect of the films containing chitosan with Cloisite 30B, and the films containing 1% and 3% of this chitosan had a log reduction that was 0.6 lower than the film with 2%. The results for these three films were very similar, although the chitosan content varied from 1-3%. A trend of higher inactivation with higher chitosan concentration was not observed. This can be caused by the uneven distribution of chitosan in the films, resulting in an equal amount of chitosan being exposed to the bacteria, or it might be because it is necessary to have a higher concentration of chitosan in the film. The observed antibacterial effect is in accordance with the results from Rhim et al. (2009), which reported a strong antimicrobial effect of Cloisite 30B against both Gram-negative and Gram-positive bacteria. The same pattern could be seen for the two films with 1% rosehip-containing chitosan and BYK tested against *E. coli*. Although one of the films contained 1% and the other 3% BYK, the log reductions were almost identical for the two films.

The results from testing of the film containing 3% unmodified chitosan (PLA/ATBC/CS3) showed that plain chitosan films had a difference in the effect against *S. aureus* and *E. coli*. There was almost a doubled effect against *S. aureus* compared to *E. coli*, with 3.25 and 1.54 log reductions respectively. These results correspond well to results found by Torlak and Sert (2013) when testing chitosan-coated polypropylene films with ISO 22196. They reported a difference in the log reduction of Gram-negative and Gram-positive bacteria of 1.04, with best effect against Gram-positive bacteria. The results from this project showed a similar difference of log 1.71. The difference might be of structural character, as the Gram-negative *E. coli* has a thicker cell wall than the Gram-positive *S. aureus*. Due to the thin cell wall of Gram-positive bacteria, the bacteria are more easily inhibited than the Gram-negative bacteria.

For *E. coli*, the log reductions for the test films with MB and PEG as plasticizers and 1-3% chitosan were very similar (log 3.06-3.67). The films with MB as plasticizer and 1% chitosan gave log reductions of 2.26-2.58, while the film with ATBC as plasticizer and 3% chitosan resulted in a log reduction of 1.54.

All tests with *S. aureus* except for one film gave invalid test results. The ISO method has strict criteria for the tests to be valid, and when the bacterial numbers on the control samples decrease too much, the results are defined as invalid. This means that the log reductions reported for *S. aureus* might not be correct, as the bacterial number in the control samples decreased with more than log 2 compared to the initial bacterial number.

The results for *S. aureus* were similar to those for *E. coli*, where the films containing both of the plasticizers MB and PEG gave the highest log reductions of 4.31-4.48. The film causing the lowest log reduction of *S. aureus* had MB as plasticizer together with unmodified chitosan and unmodified nanoclay, resulting in a log reduction of 1.73. By addition of 3% copolymer (BYK) to this film, the antibacterial effect was almost doubled against *S. aureus* (log reduction of 2.98).

The ISO method has been used at the laboratory at Nofima for several tests of antibacterial plastic films. The experience, including the results from this master project, show that it has been difficult to obtain reproducible results. The films are composed of various materials, and different surface properties between the test film and the non-active control film occur. The results of validated tests can be used as a positive documentation, but alternative methods were therefore needed to document the antimicrobial effect for food packaging applications.

It is likely that the uneven distribution of the chitosan in some of the films affected the results, and a different film (PLA/ATBC/CS5) was therefore used for the following experiments. This film had the chitosan evenly spread, and it had no air bubbles within the film structure. It was used a higher chitosan concentration (5%) in order to detect an antibacterial effect of the chitosan, compared to the other films tested which contained 1-3% chitosan.

4.2 Test of chitosan in liquid solution

4.2.1 Chitosan-containing film

In these experiments, different amounts of the chitosan-containing film PLA/ATBC/CS5 were tested. Chitosan films of 10 or 25 cm^2 were cut in pieces and added to tubes containing 25ml nutrient broth with an overnight culture of bacteria. Tubes without chitosan film were used as control. The purpose of testing the films was to evaluate the potential for use in foods; therefore rich media was used in these experiments. The bacterial numbers and standard deviations for these experiments are shown in appendix A-2.

The initial number of bacteria was approximately log 7 cfu/ml and the samples were incubated at 30°C and 70rpm for 24h. The rich media and high temperature gave a rapid growth, and the bacterial numbers in all samples increased with 1.5-2.0 log units (Figure 4.2). A lower increase in the bacterial number in the chitosan-containing samples was expected. The high density of bacteria in the solution might create a situation where only a few bacteria are in contact with the chitosan in the tube, even with shaking of the growth tubes. The bacteria that are not in contact with the chitosan will grow quickly, resulting in a total increase in bacterial number. Additional experiments were therefore started with a lower initial bacterial number, so that a higher percentage of the total bacterial number would be in contact with the chitosan.

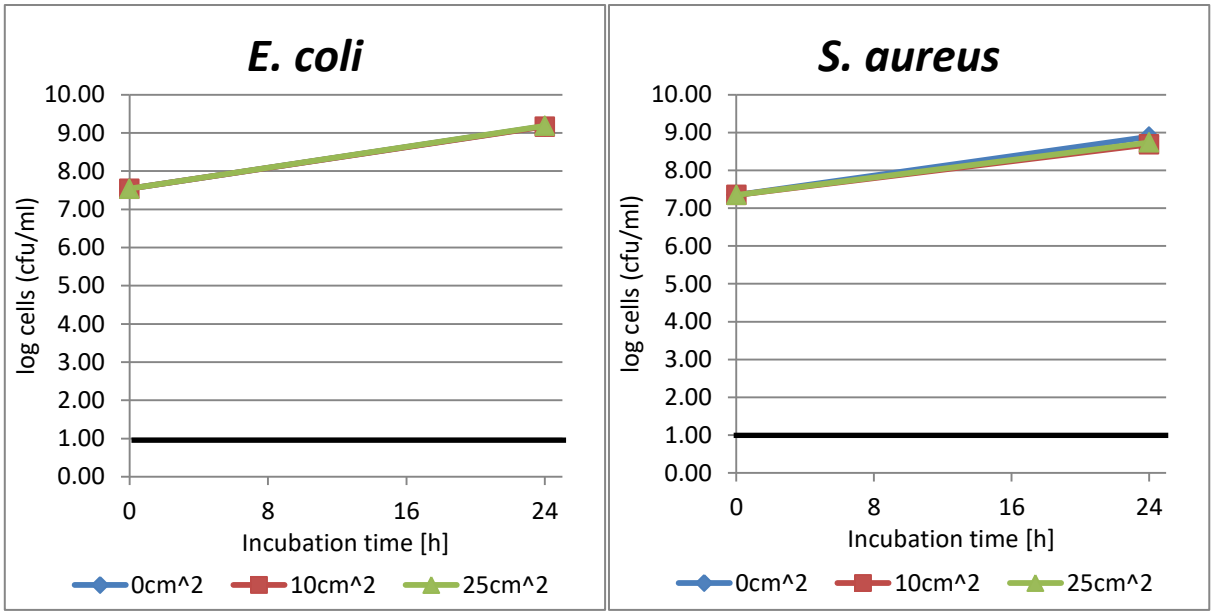

Figure 4.2: Bacterial counts after incubation at 30°C with chitosan film, starting with a high bacterial number. All sampling were performed in triplicate. The error bars represent standard deviation, and the solid black line indicates the detection limit.

The results from the experiments with an initial number of bacteria at approximately log 5 cfu/ml and incubation for 24h at 30°C and 70rpm (Figure 4.3) showed no inhibitory effect of chitosan on *E. coli*, where the bacterial numbers in all samples increased with about 2 log units. For *S. aureus* there was also an increase in the numbers of about 2 log units for the control sample and the sample with 10cm^2 chitosan film. For the sample with 25cm^2 film, there was a reduction of 1.5 log units. This result is very similar to the results reported by Turalija et al. (2016), who found no antibacterial effect of a film containing PLA and 10% chitosan. The results are also in accordance with the results found when testing PLA/ATBC/CS3 by ISO 22196, where *S. aureus* was more sensitive to chitosan films than *E. coli*. Rhim et al. (2006) however, reported a 3 log reduction of both *E. coli* and *S. aureus* with the same test method and conditions. This could indicate that the method is not stable enough to be used alone as documentation for antibacterial activity of chitosan films.

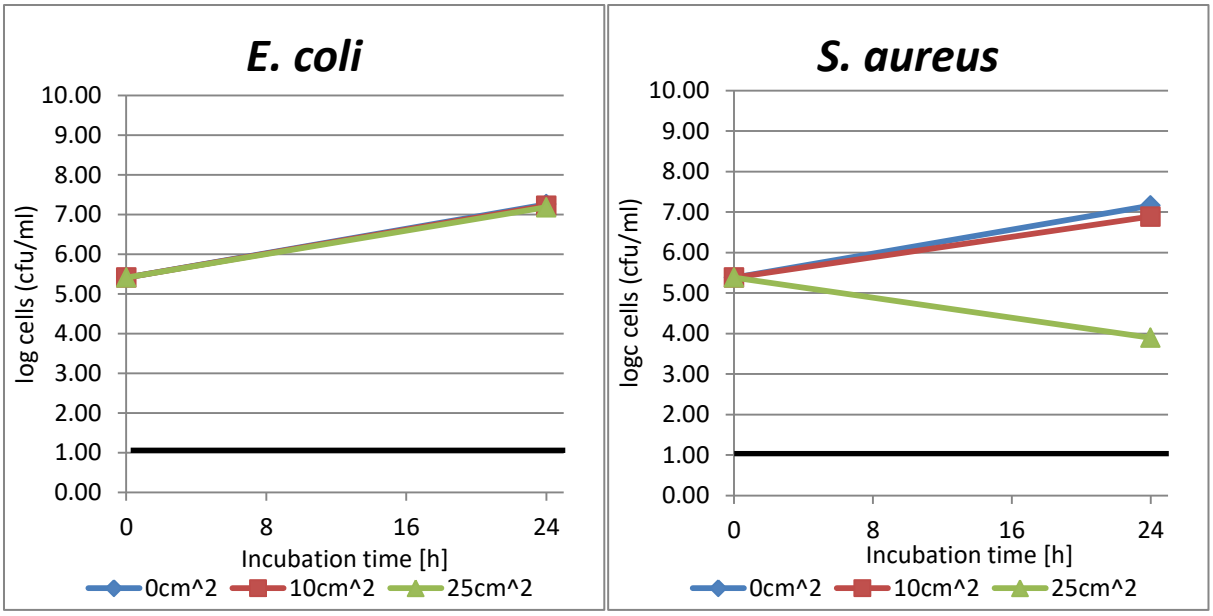

Figure 4.3: Bacterial counts after incubation at 30°C with chitosan film, starting with a low bacterial number. All sampling were performed in triplicate. The error bars represent standard deviation, and the solid black line indicates the detection limit.

The incubation temperature used enhances rapid growth of the bacteria. A lower incubation temperature is more realistic in relation to the use of the films for packaging material on chilled food. The food safety authority in Norway requires perishable food to be stored at ≤4°C ("Forskrift om næringsmiddelhygiene," 2009). Permanent or occasional temperature abuse is well known in the Norwegian chill chain, and e.g. the transport between stores and consumers can cause the temperature to increase far above the limits. The samples were therefore incubated at 10°C, which is within a relevant temperature for some foods.

Starting with a high initial bacterial number of log 7 cfu/ml (*E. coli*) and log 8 cfu/ml (*S. aureus*), followed by incubation at 10°C and 70 rpm with sampling after 0, 2, 5, 7 and 9 days of incubation, there were not detected an antibacterial effect of the chitosan film (Figure 4.4). All samples with *E. coli* had an increase in bacterial counts during the first five days of incubation, before the counts were stabilized at log 8.5 cfu/ml. For the samples with *S. aureus*, the bacterial counts decreased slowly during incubation in all samples, ending at a final count just below log 7 cfu/ml. The next experiments were therefore conducted with a start concentration of bacteria at approximately log 5 cfu/ml in order to see if the effect of chitosan was better with fewer bacteria.

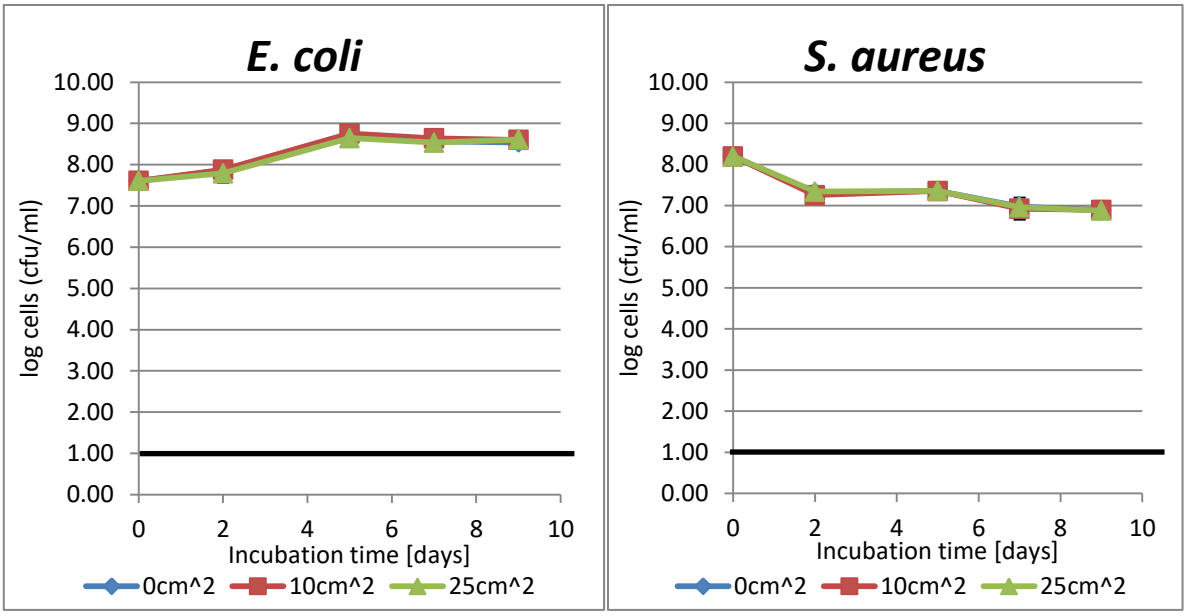

Figure 4.4: Bacterial counts after incubation at 10°C with chitosan film, starting with a high bacterial number. All sampling were performed in triplicate. The error bars represent standard deviation, and the solid black line indicates the detection limit.

In the experiments with a lower start number of bacteria (log 5) and incubation at 10°C and 70 rpm, there was a clear antibacterial effect of chitosan on both of the tested bacteria during the 14 days of incubation (Figure 4.5). For *E. coli*, the bacterial counts decreased with increasing chitosan concentration. The samples without chitosan had a total increase in bacterial counts of approximately log 1 during the sampling period, while the number in the samples containing chitosan decreased. The sample with 10cm^2 film had a total decrease of log 2, while in the sample with 25cm^2 film the counts decreased below the detection limit (log 1) after five days of incubation. This is an inactivation of more than 4 log units. For *S. aureus* on the other hand, the bacterial counts decreased in all samples. In the control sample it decreased approximately log 1.5, while in all chitosan-containing samples, regardless the chitosan concentration, the detection limit was reached between five and seven days of incubation.

The high standard deviation for *E. coli* samples with 10cm^2 film was caused by the three parallel samples having different bacterial numbers after incubation. Two of the samples had bacterial counts about log 5, while the last sample had no detectable growth from day five until the end of incubation. At the sampling performed on day five of incubation, the *S. aureus* sample with 10cm^2 had a standard deviation of 1.40. This was because two of the parallels had counts at log 2, while the third parallel had no detectable growth. It is not known what cause these differences between parallels.

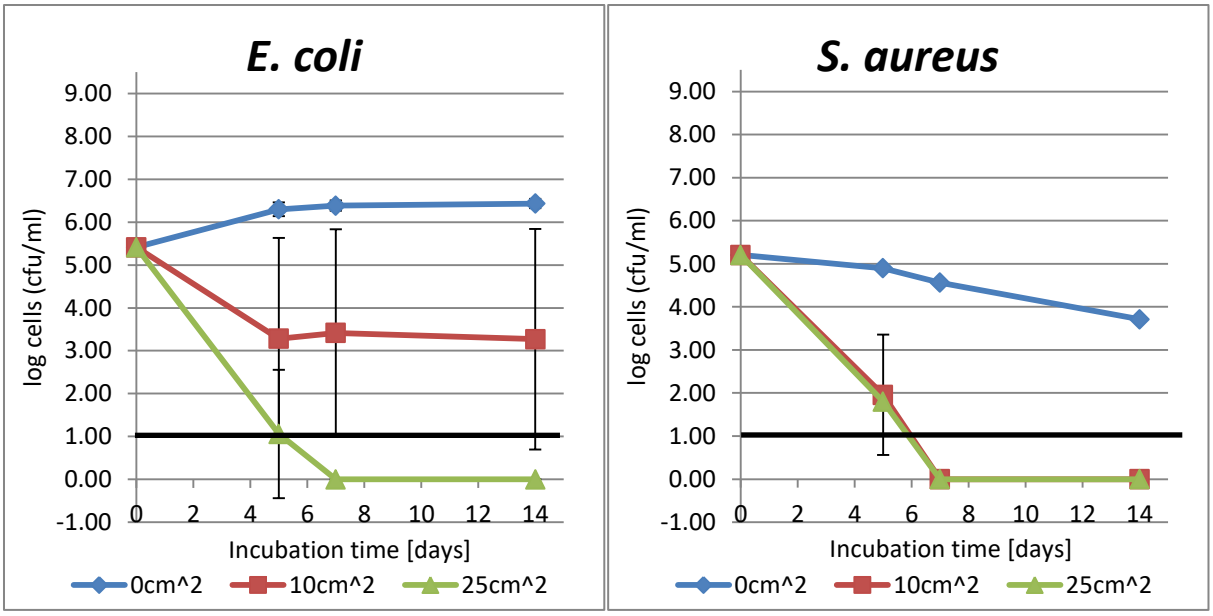

Figure 4.5: Bacterial counts after incubation at 10°C with chitosan film, starting with a low bacterial number. All sampling were performed in triplicate. The error bars represent standard deviation, and the solid black line indicates the detection limit.

These results showed that if the bacterial numbers were very high in the solution, the chitosan film affected few bacteria and no detectable inactivation was observed. They also showed that stressed bacteria were less resistant against the antibacterial effect of chitosan, as described by Randazzo et al. (2016). Low temperature and presence of chitosan are two factors that stress the bacteria. The results indicate that *S. aureus* is more sensitive to chitosan than *E. coli*. When the samples were incubated at 10°C starting with a bacterial number about log 5 cfu/ml, the bacterial counts in all chitosan-containing test tubes decreased below the detection limit for *S. aureus*, while only the samples with 25cm^2 film decreased below the detection limit for *E. coli*. None of the other incubation temperatures or bacterial numbers gave samples that decreased below the detection limit during incubation. In order to see if the antibacterial effect of chitosan decreases when it is encapsulated in a rigid film structure, experiments were conducted with chitosan powder.

4.2.2 Chitosan powder

For testing of chitosan powder, the same method that was used to test film in liquid solution was selected. The amounts of chitosan powder were calculated to be the same amount as in the film previously tested. These amounts were found by weighing 25cm^2 chitosan film and calculation of the weight equivalent to 5% of the film. The calculated amount was then equivalent to the chitosan in 25cm^2 film, and similar calculation was used for 10cm^2 film. The calculated amounts of chitosan powder were 20mg corresponding to 25cm^2 film, and 8mg corresponding to 10cm^2 film. Tubes without chitosan powder were used as control. Values for bacterial numbers and standard deviations for the experiments performed with chitosan powder are shown in appendix A-3.

Experiments were first carried out with an initial bacterial number of about log 7 cfu/ml, with incubation at 30°C and 70rpm. The results (Figure 4.6) showed that there was an effect on *E. coli* towards the end of the incubation. The graphs for all chitosan concentrations were similar during the first eight days of incubation, but from eight to fourteen days there was a decrease of log 1 for the 20mg samples and about log 0.5 for the 8mg samples. A replicate of the experiment with longer

incubation time would have been appropriate to see if this decrease can be related to the chitosan concentration. For *S. aureus* there was no effect during the incubation period. Both bacteria had an increase of about log 1.5 from day zero to day two, which is a similar growth that was detected when testing chitosan film under these same conditions.

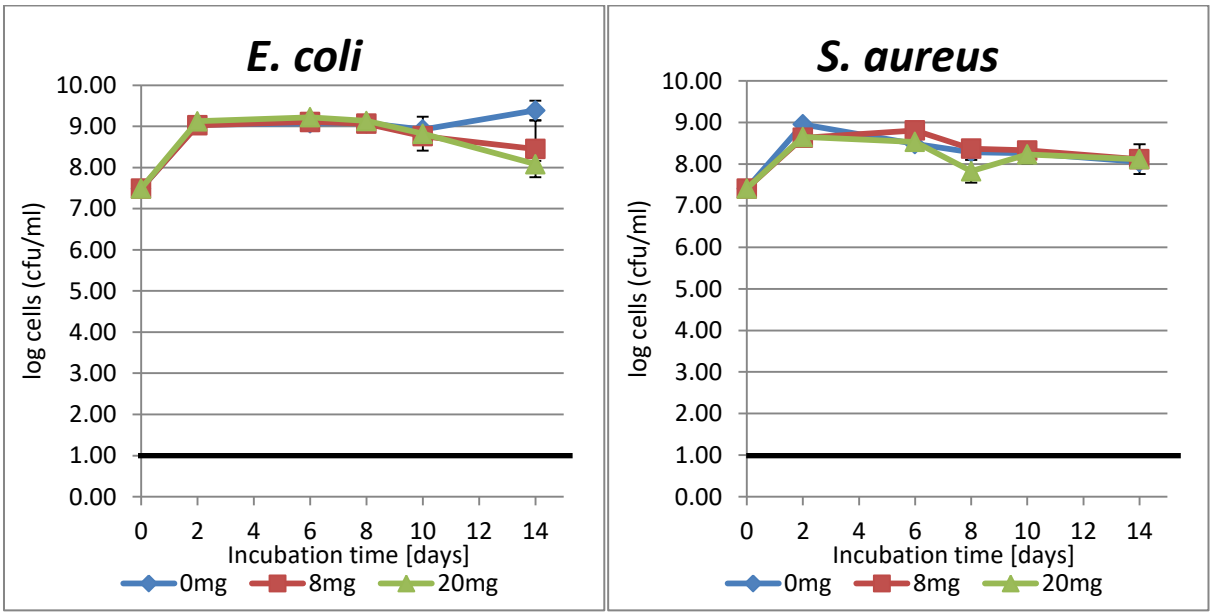

Figure 4.6: Bacterial counts after incubation at 30°C with chitosan powder, starting with a high bacterial number. All sampling were performed in triplicate. The error bars represent standard deviation, and the solid black line indicates the detection limit.

The results at 30°C with a high start number of bacteria were very similar to the results obtained when testing chitosan film, and a lower initial bacterial number was therefore tested with the same amounts of chitosan powder at 30°C and 70rpm. These results (Figure 4.7) showed that the chitosan powder had a stronger antibacterial effect than the film in the bacterial broth. Almost no effect for any amount of film was found against the bacteria under these conditions, while the powder had a clearly detectable effect against both bacteria. The reduction in the bacterial count increased with higher amount of chitosan powder present.

For *E. coli* the samples with 8mg chitosan powder had standard deviation close to log 2.6 from day 6-14. The three parallel samples had very different counts; one of the parallels had counts at log 5, one of the samples had counts at log 3, while the last sample did not have detectable growth. Comparable variation was found for *S. aureus*; the 8mg samples had a standard deviation of log 1.47 at day six and log 1.85 at day 14, while the 20mg samples had a standard deviation of log 1.13 at day eight and log 1.18 at day ten. At the other sampling times the standard deviations were below 0.45. It is important to notice that the control samples for both bacteria had low standard deviations, while growth tubes with bacteria and chitosan varied from below detection level in some samples to log 1-5 in other samples. The reason behind these large differences in inactivation is not documented, but as discussed with the chitosan films (4.2.1) the combination of stress factors might lead to the variation in inactivation. Also the combination with low initial bacterial numbers influences the inactivation levels.

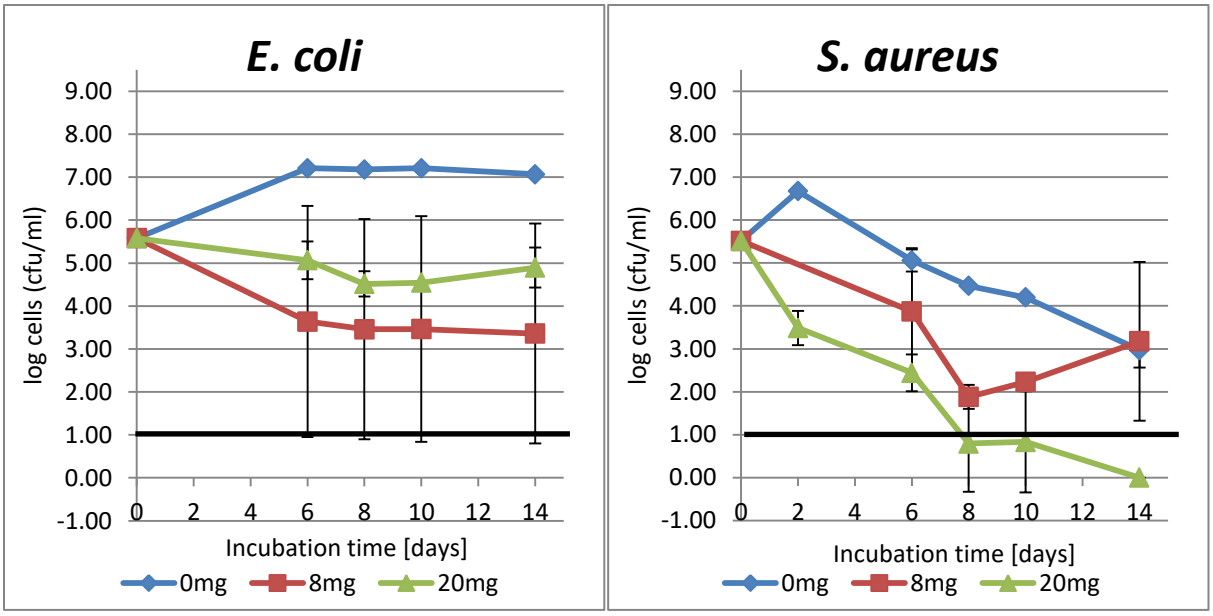

Figure 4.7: Bacterial counts after incubation at 30°C with chitosan powder, starting with a low bacterial number. All sampling were performed in triplicate. The error bars represent standard deviation, and the solid black line indicates the detection limit.

It was decided to examine if there was a better antibacterial effect at lower temperature, and experiments were performed with 10°C incubation. Starting with a bacterial number at log 7 cfu/ml, there was no detectable effect on the counts for *S. aureus*, while for *E. coli* there appeared to be an effect at day two (Figure 4.8). At day two the chitosan-containing samples had a bacterial count at log 6 and the control sample had a count at log 8, but throughout the incubation period the bacterial counts for the chitosan-containing samples increased until they stabilized at log 8.5, at the same level as the control sample. There was no difference between the samples for *S. aureus* during the incubation period; all samples had stable enumerations at log 6.9-7.6. This means that the results for incubation at 10°C with a high start number of bacteria was equal for the powder as they were for the film.

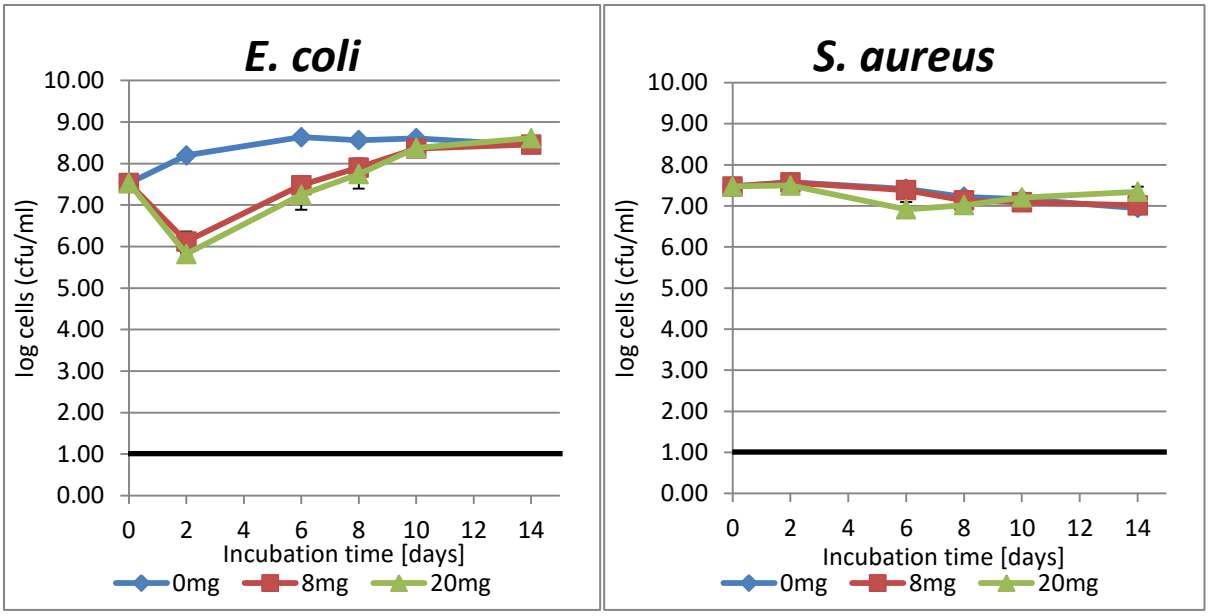

Figure 4.8: Bacterial counts after incubation at 10°C with chitosan powder, starting with a high bacterial number. All sampling were performed in triplicate. The error bars represent standard deviation, and the solid black line indicates the detection limit.

The highest antibacterial effect of chitosan was seen when testing chitosan powder, starting with a low bacterial number and incubation at 10°C (Figure 4.9). Here, *E. coli* decreased below the detection limit between two and six days of incubation for both chitosan concentrations. Compared to the testing of chitosan film, the inactivation effect with chitosan powder was more powerful. For the film, only the sample with 25cm^2 decreased below the detection limit. For *S. aureus*, the numbers decreased below the detection limit close to two days of incubation for these samples. It took almost three times longer for the film samples than for the powder samples before the numbers were below log 1.

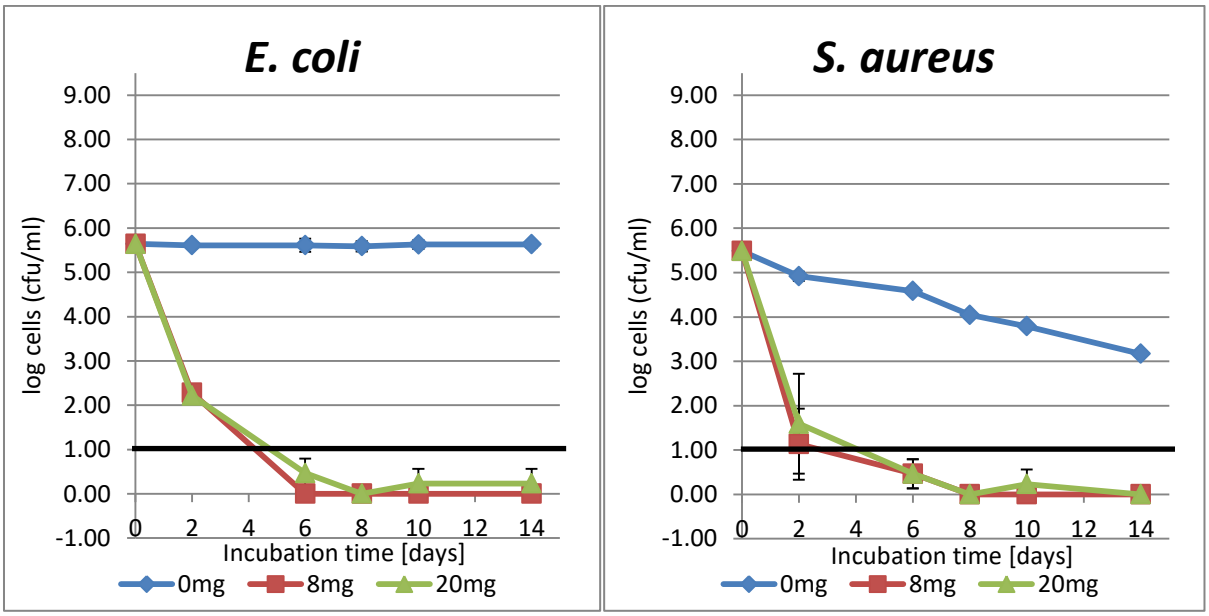

Figure 4.9: Bacterial counts after incubation at 10°C with chitosan powder, starting with a low bacterial number. All sampling were performed in triplicate. The error bars represent standard deviation, and the solid black line represents the detection limit.

An objective with the broth experiments was to document how different concentrations of chitosan affected inactivation of bacteria in an environment that simulate situations in a food packaging system. The *in situ* experiments only mimic some of the physical and chemical factors in food packaging, but still revealed some important challenges with inactivation using components that need contact with the bacteria to give effects. Based on all results from testing of film and powder by the same method and at the same conditions, the antibacterial effect of chitosan appeared to be reduced when chitosan was embedded in a packaging film. The chitosan powder showed effect against both *E. coli* and *S. aureus* at both 30°C and 10°C when the start number of bacteria was about log 5 cfu/ml, while the chitosan film only showed a small effect at 10°C and no effect at 30°C. The results also showed that the best effect of chitosan was obtained when the bacteria were exposed to several combined stress factors, in this case by incubating a low bacterial number at low temperature and exposure to chitosan.

Based on the inactivation results from testing of chitosan powder, it was decided to examine chitosan colloid which is chitosan powder dissolved in acetic acid. However, in order to obtain a high concentration of dissolved chitosan a very strong acid has to be used. Most likely the bacterial growth would then be inhibited by the low pH, and not by the chitosan. Therefore, the chitosan colloid was not tested in growth tubes as described in the previous experiments. Instead it was decided to examine the growth curves with dissolved chitosan at specific chitosan concentrations and pH values. The growth curve of samples containing chitosan colloid compared to samples without chitosan were analyzed by use of optical density (OD) measurements.

4.3 Growth curve analyses

Before testing of chitosan colloid, growth curves were made for both test strains at eight concentrations prepared by serial dilution, in addition to a negative control. These curves were then used to determine the time to detection (TTD) in order to make a calibration curve to control if there was a linear relation between OD and bacterial concentration. Values for bacterial numbers and standard deviations are shown in appendix B.

The growth curves for *E. coli* (Figure 4.10) showed that the samples with higher bacterial concentrations gave detectable growth earlier than the samples containing fewer bacteria. The detection limit was set to OD_{600}=0.2, as all samples had initiated exponential growth when reaching this OD. The sample with a bacterial concentration of log 8 cfu/ml was excluded from the further calculations, as this sample had OD_{600}=0.325 at the first measurement, which was above the detection limit.

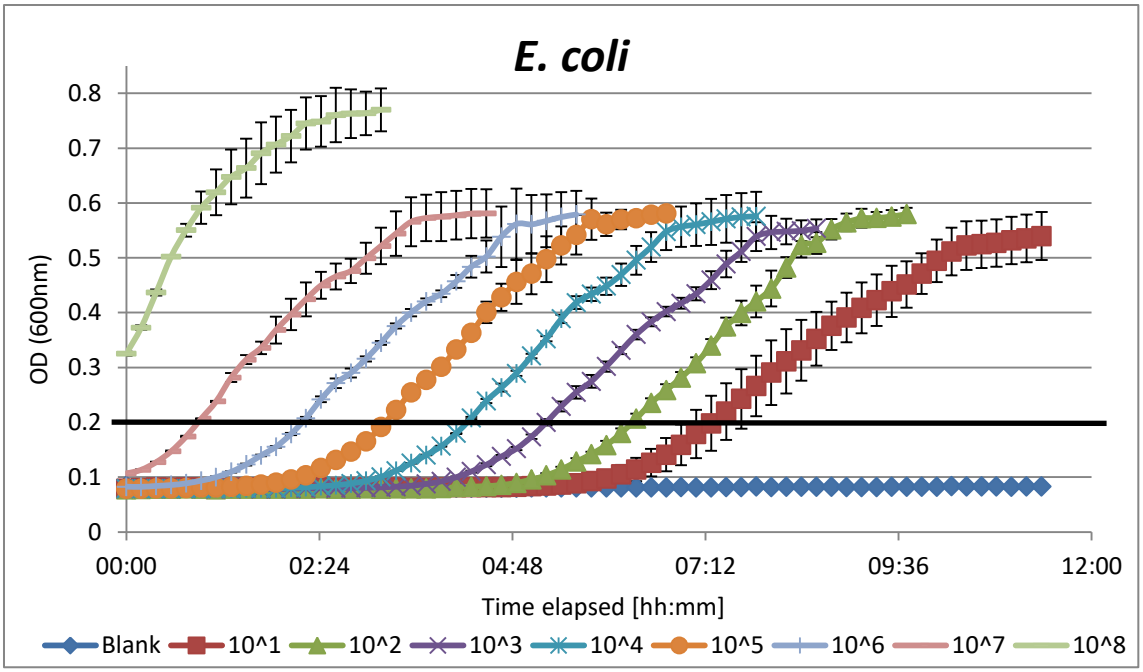

Figure 4.10: Growth curves of different concentrations of *E. coli*. All data points are averages of three parallel samples, with error bars representing standard deviation. The solid black line represents the detection limit, OD_{600}=0.2.

The growth curves for *S. aureus* (Figure 4.11) showed the same correlation between TTD and initial bacterial concentration. Here as well the detection limit was set to OD_{600}=0.2, and in the same way as for *E. coli*, the sample with an initial bacterial concentration of log 8 cfu/ml was excluded from the calculations as it started with an OD above the detection limit (OD_{600}=0.418).

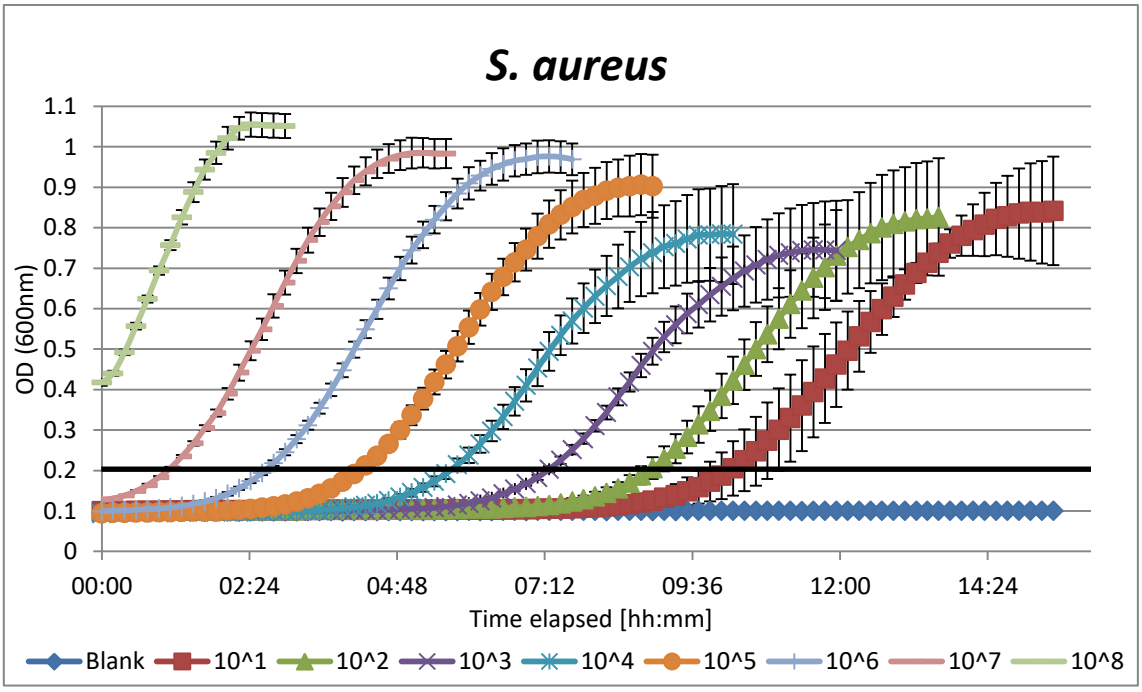

Figure 4.11: Growth curves of different concentrations of *S. aureus*. All data points are averages of three parallel samples, with error bars representing standard deviation. The solid black line represents the detection limit, OD_{600}=0.2.

Linear regression of the exponential growth period was used to find a formula (y=ax+b) by which TTD was calculated. TTD for the different bacterial concentrations are given in Appendix B-1. TTD and bacterial concentrations were used to make calibration curves for the two bacteria (Figure 4.12). Both curves show a good correlation between OD_{600} and bacterial concentration, as R^2>0.998 for the linear trend lines.

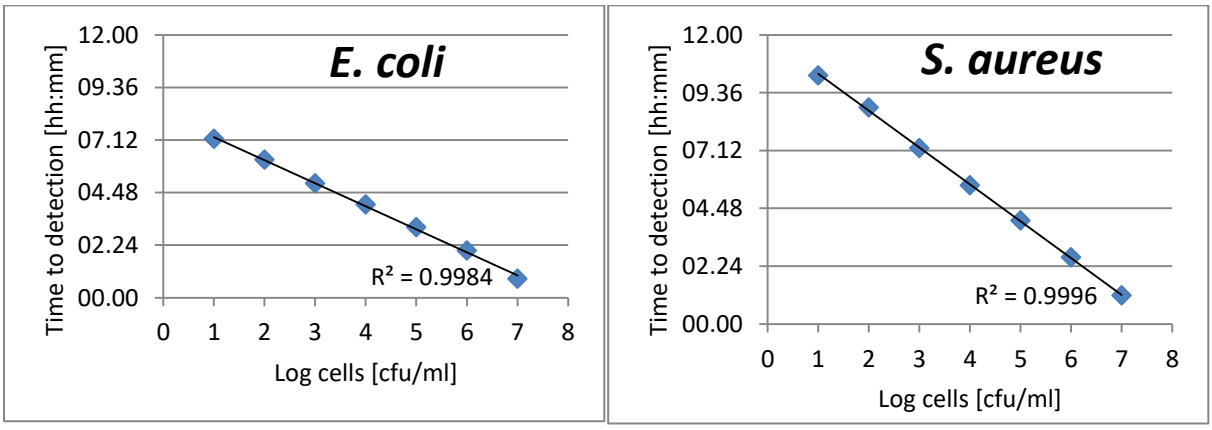

Figure 4.12: Calibration curves for *E. coli* and *S. aureus* with detection limit at OD_{600}=0.2

The fact that the calibration curves have a good fit to a straight line indicates that the broths used are suited for the respective bacteria (Johnston, 1998). The calibration curves can be used for later experiments performed under equal conditions to calculate the bacterial concentration in a sample by use of the TTD for the sample.

4.3.1 Chitosan colloid

According to Yılmaz Atay & Çelik (2017), dissolved chitosan is a better antibacterial agent than chitosan powder and chitosan-containing films. This is because the antibacterial effect of chitosan powder and film is limited to those bacteria that are in contact with the chitosan grains, while chitosan that is dissolved in a liquid is present in the entire solution as long as it is stirred regularly. With previous experiments showing good effect of chitosan film and chitosan powder against low bacterial concentrations at low temperature (10°C), chitosan was dissolved in acetic acid to see if it was possible to detect an effect on the growth curves.

Solution prepared with 1% acetic acid

First, a solution with a concentration of 1% acetic acid was used in order to completely dissolve the chitosan powder. However, this high concentration of acid resulted in a pH about 5.0 in the final solution, which is close to the lower pH limit for growth for both test bacteria. This was confirmed by the results for both bacteria, where no growth was detected for any samples except the control sample (Figure 4.13). It is likely that the inhibition was caused by the low pH caused by the acid in which the chitosan powder had been dissolved.

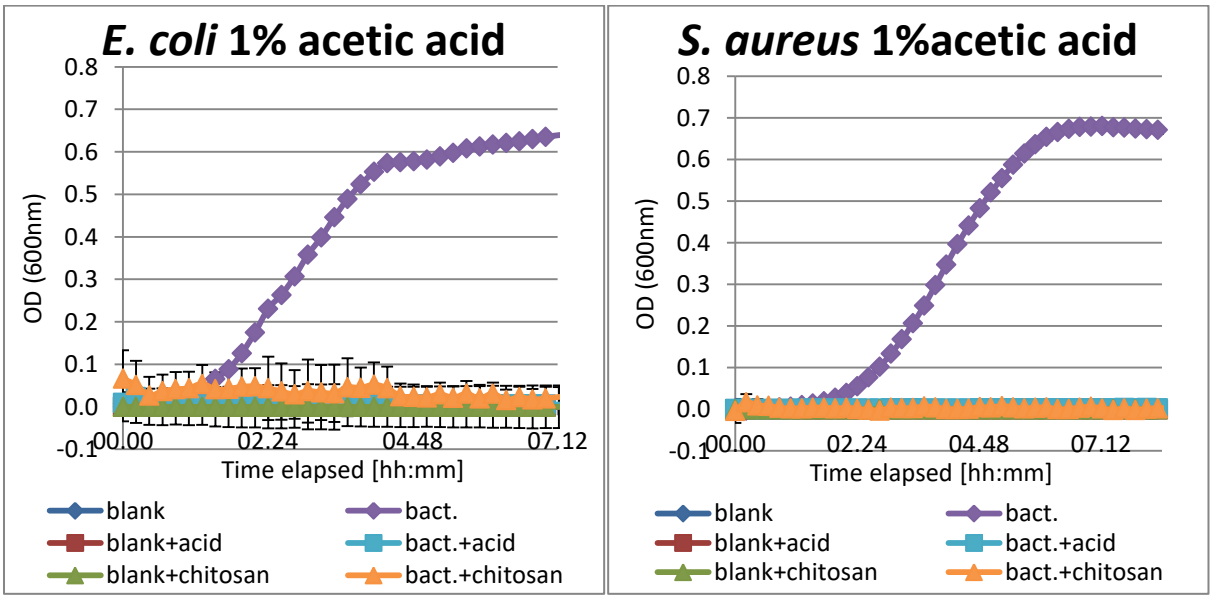

Figure 4.13: *E. coli* and *S. aureus* growth curves in TSB or BHI (blank), respectively, with and without chitosan colloid and 1% acetic acid. The bacterial concentration in the test samples was log 6 cfu/ml. All data points are averages of three parallel samples, with error bars representing standard deviation.

Solution prepared with 0.1% acetic acid

In order to avoid growth inhibition caused by low pH, it was decided to test an acid concentration of 0.1%. Preparation of chitosan colloid and test solutions by use of 0.1% acetic acid gave a pH≈6.8 in the test solutions, which should not cause inactivation of the bacteria. However, the chitosan was only partially solubilized by this acid causing a chitosan concentration in the test solutions slightly lower than for the experiments with 1% acetic acid. The undissolved chitosan powder remained as solid particles in the solution, and some chitosan powder was possibly not transferred to the test wells.

The result from testing of 0.1% acetic acid and chitosan colloid prepared with 0.1% acid against *E. coli* is shown in Figure 4.14. The detection limit was set to OD_{600}=0.1, because the samples had reached exponential growth when they reached this OD. The control sample with bacteria had TTD=2h 3min, and the solution with acetic acid had TTD=2h 16min. As the difference between the TTD between these samples was 13min, one can conclude that the acetic acid, at this low concentration, only caused a minor growth inhibition of *E. coli*. The sample with bacteria and chitosan colloid, however, had TTD=10h 56min, an extended time of 8h 53min compared to the control sample. Also the growth rate was slower and the density of bacteria (max OD) was lower for the sample with bacteria and chitosan compared to the samples with bacteria and water or acid.

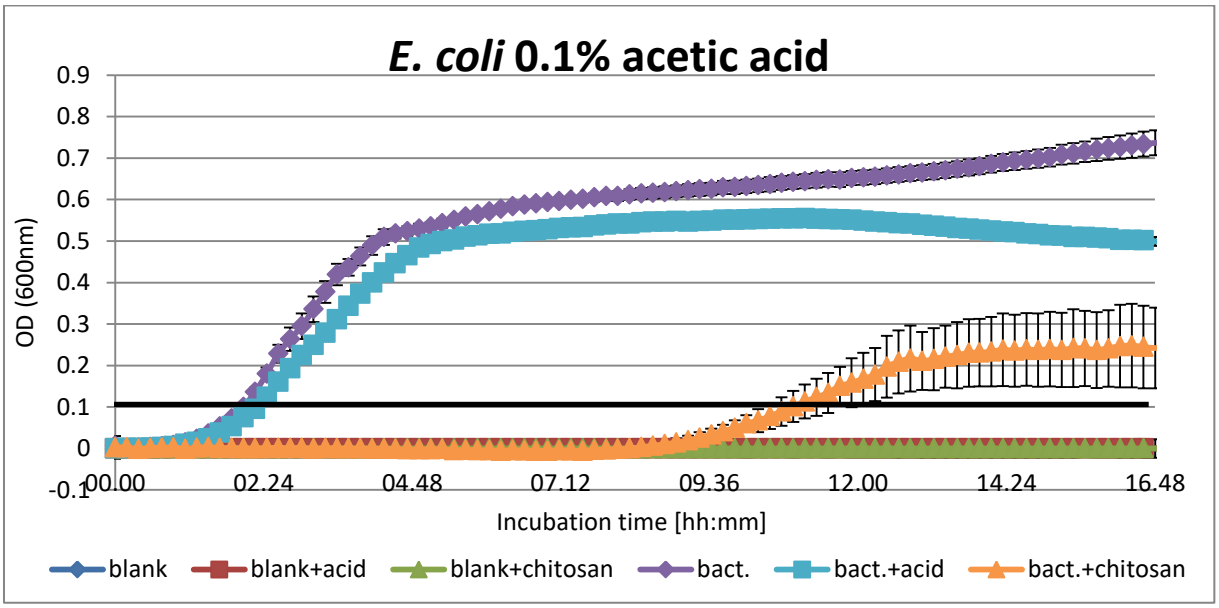

Figure 4.14: *E. coli* growth curves in TSB (blank) with and without chitosan colloid and 0.1% acetic acid. The bacterial concentration in the test samples was log 6 cfu/ml. All data points are averages of three parallel samples, with error bars representing standard deviation. The solid black line represents the detection limit, OD_{600}=0.1.

Similar trends that were observed for *E. coli* were observed for *S. aureus* (Figure 4.15). Here, the difference in TTD for the bacteria sample with water and with acetic acid was 16min, also a minor growth inhibition from the acetic acid. The major difference between the results from testing of the two bacteria was that for *E. coli* the chitosan-containing sample reached the detection limit 8h 53min after the control sample, while for *S. aureus* it reached the detection limit only 1h 19min after the control sample. This indicates that in this experimental design the Gram-positive *S. aureus* is less sensitive to chitosan compared to the Gram-negative *E. coli*.

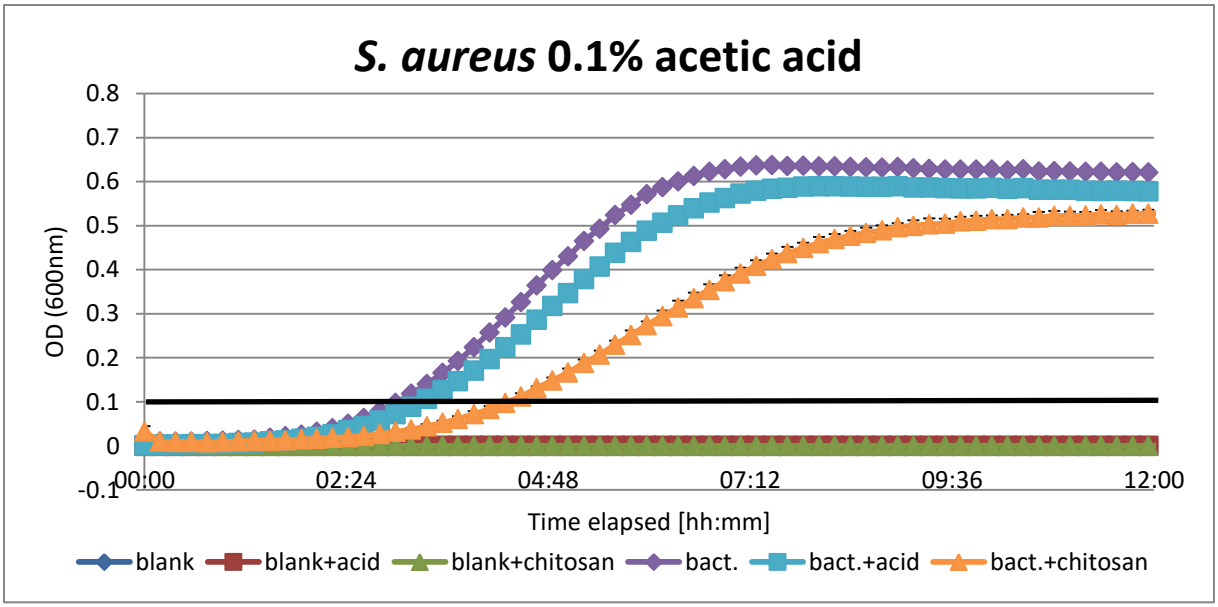

Figure 4.15: *S. aureus* growth curves in BHI (blank) with and without chitosan colloid and 0.1% acetic acid. The bacterial concentration in the test samples was log 6 cfu/ml. All data points are averages of three parallel samples, with error bars representing standard deviation. The solid black line represents the detection limit, OD_{600}=0.1.

The results from these experiments show that the test strains were only minimal inhibited by the acid, and a delay in the growth curve was observed for both bacteria when chitosan colloid was added to the samples. In the chitosan-containing samples, it took longer time to reach the detection limit for both bacteria, and the growth rate was slower compared to the samples without chitosan. Based on the low growth inhibition by the acid, it was decided to conduct experiments with 0.5% acetic acid to see if this acid could dissolve the chitosan completely, without inhibiting the bacterial growth.

Solution prepared with 0.5% acetic acid

The 0.5% acetic acid solution almost completely dissolved the added chitosan powder, leaving a few particles visible in the solution. It gave a pH of 5.5 in the test solutions. This pH is slightly higher than the pH in the test solutions when 1% acetic acid was tested, but it is still above the minimal pH for growth of the test bacteria.

The results from testing of 0.5% acetic acid against *E. coli* showed that this concentration of acetic acid completely inhibited the bacterium during the incubation time (Figure 4.16). The bacteria were inhibited by the low pH caused by the acid in the solution, and hence one cannot detect an antibacterial effect of chitosan from this result.

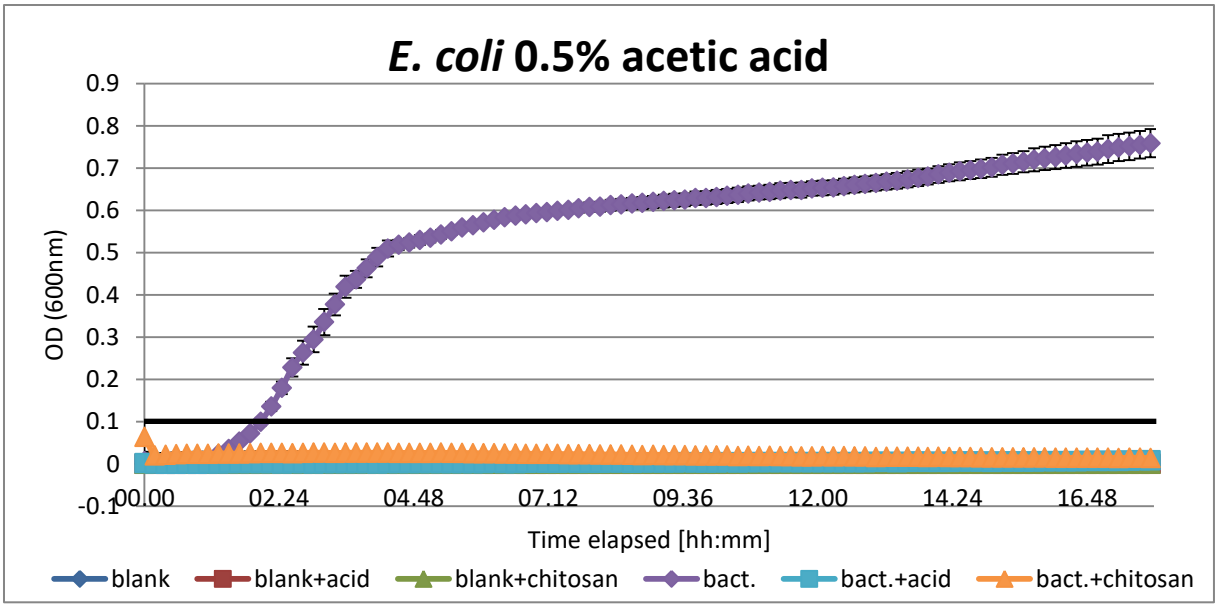

Figure 4.16: *E. coli* growth curves in TSB (blank) with and without chitosan colloid and 0.5% acetic acid. The bacterial concentration in the test samples was log 6 cfu/ml. All data points are averages of three parallel samples, with error bars representing standard deviation. The solid black line represents the detection limit, OD_{600}=0.1.

The results from testing of *S. aureus* at the same conditions showed that, in contrast to *E. coli*, this bacterium was not inhibited by the acetic acid (Figure 4.17). The sample containing acetic acid reached the detection limit 4h after the sample with only bacteria, showing that the acid partly inhibits the bacteria. Both samples had exponential growth, meaning that the bacteria were not completely inhibited. When growth was initiated, the growth rate was lower compared to the control sample.

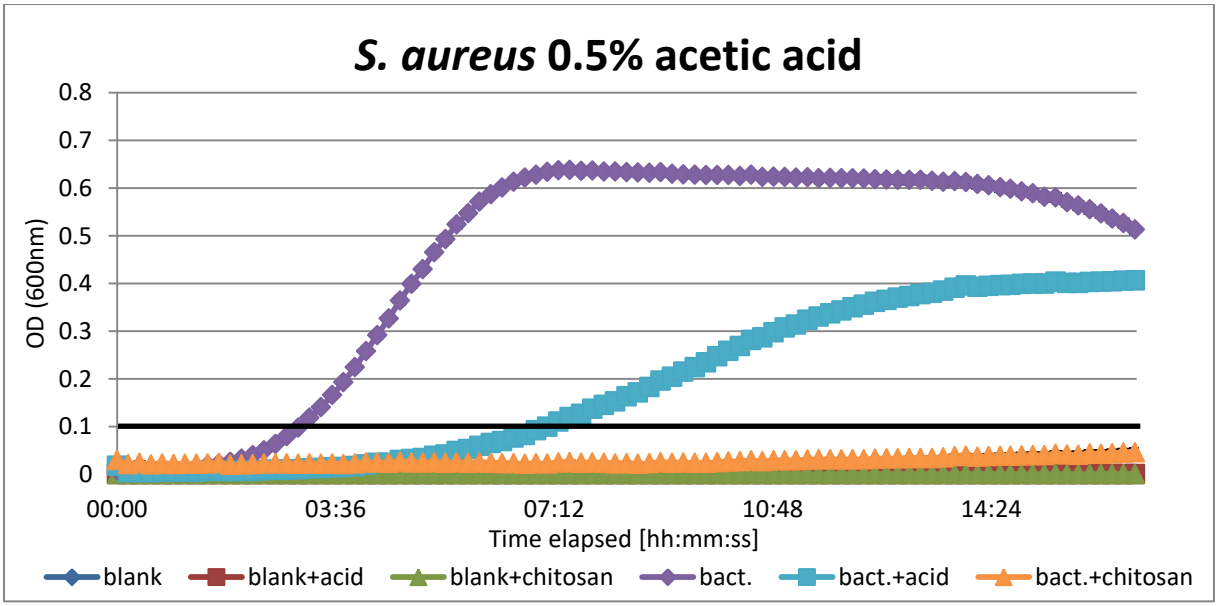

Figure 4.17: *S. aureus* growth curves in BHI (blank) with and without chitosan colloid and 0.5% acetic acid. The bacterial concentration in the test samples was log 6 cfu/ml. All data points are averages of three parallel samples, with error bars representing standard deviation. The solid black line represents the detection limit, OD_{600}=0.1.

The results showed that *E. coli* was completely inhibited by 0.5% acetic acid for the entire incubation period, while *S. aureus* was not. For *S. aureus*, the acid caused a delay in TTD of 4h compared to the control sample, while the chitosan colloid gave a growth curve that never exceeded the detection limit.

The results showed that chitosan had an antibacterial effect, and that the degree of inhibition varied between the tested bacteria. In these experiments *E. coli* was more easily inhibited by chitosan than *S. aureus*, which has previously been reported by other researchers (Kong et al., 2010; Sánchez-González, Cháfer, Hernández, Chiralt, & González-Martínez, 2011). There was also a different acid tolerance between the two test strains. *E. coli* had a much longer TTD at a lower concentration of acetic acid than *S. aureus*.

The growth curves obtained with the Bioscreen OD measurements compliment a picture of how chitosan functions as an antimicrobial compound in a complex matrix. In the experiments with chitosan embedded in films and chitosan powder, it is the combined hurdle effects that give an inhibition. Dissolved chitosan need a solvent, in this case acetic acid, which reduce the pH. The effect of pH is clearly shown, but the effect of partly dissolved chitosan in the 0.1% acid samples also showed a clear inhibitory effect of chitosan. The antibacterial effect of chitosan is affected by many environmental factors such as pH, temperature, and contact time between bacteria and chitosan (Alishahi & Aïder, 2012). It is worth noting that, despite the widely reported antimicrobial properties of chitosan in the literature, the results are mainly based on *in vitro* experiments. In real-world applications it is important to consider that most foods are complex matrices composed of different compounds (proteins, carbohydrates, lipids, minerals, vitamins, salts, and others), and many of them might interact with chitosan to varying levels, possibly leading to a loss or enhancement of its antibacterial activity (Devlieghere et al., 2004).

4.4 Experiments with food matrix

The food matrix used in these experiments was smoked salmon. The control samples were packaged in a neutral tray (HDPE), and the test samples were packaged in a PLA-chitosan tray that was placed inside a neutral tray (Figure 3.5B). All samples were flushed with 100% nitrogen before they were sealed in order to simulate vacuum packaging which is normal for smoked salmon. The samples were stored at 4°C to day 27, and then the temperature was increased to 8°C from day 27 to day 41. Values for bacterial numbers and standard deviations are shown in appendix A-4.

For the Iron agar plates, the bacterial number at day zero was between log 1 and log 2 (Figure 4.18 and Figure 4.19). This show that the salmon raw material was of good microbial quality. The number of H_2S-producing bacteria which make black colonies was low, and the two figures, showing total viable count and white colonies, are almost identical during the storage period. The microbial evaluation of the spoilage numbers was therefore based on Figure 4.19, which shows total viable count on Iron agar.

The bacterial counts for the control sample increased to log 3.25 at day 15. From this day and until the last sampling, at day 41, there were only small differences between the bacterial numbers, and the sample ended at log 3.39 at day 41. At day 15, the bacterial numbers in the control and the PLA-chitosan tray were very equal. Until day 27, the samples packaged in chitosan trays had a bacterial count about log 3, while at day 34 and 41 the bacterial number was about log 5. This is more than 1.5

log units higher than the mean numbers in the control samples, indicating that there was no inhibitory effect of the chitosan in the packaging material.

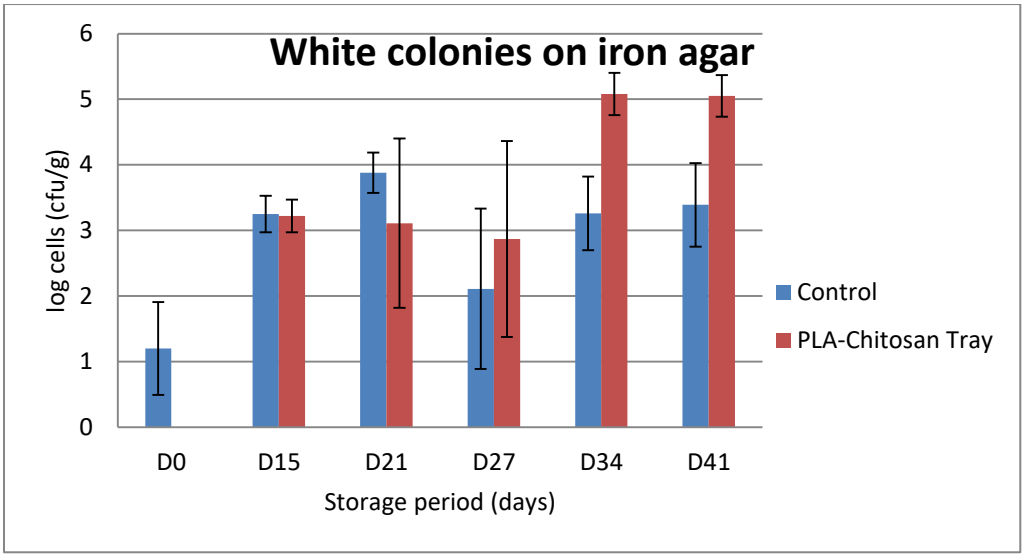

Figure 4.18: White colonies measured in log (cfu/g) on Iron agar. Sampling was performed in triplicate, and the error bars represent standard deviation.

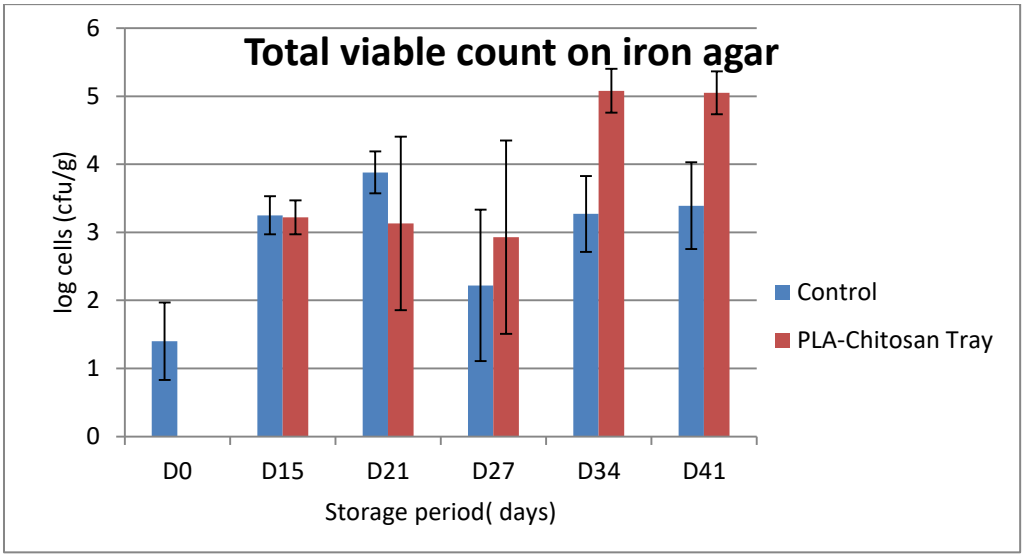

Figure 4.19: Total viable counts measured in log (cfu/g) on Iron agar. Sampling was performed in triplicate, and the error bars represent standard deviation.

The number of psychrotrophic bacteria (Figure 4.20) was comparable to the numbers found on Iron agar. The standard deviations at all sampling times were relatively high, indicating that there were no differences between the two packaging materials until day 27. From day 27 to day 41 the numbers from the PLA-chitosan tray were higher than for the control samples.

Spread plating on Long & Hammer agar allowed detection of psychrotrophic and heat labile microorganisms. Bacteria that are heat labile will not be detected on Iron agar, as they will die in the 45°C agar that is needed to make pour plates. Psychrotrophic and heat labile microorganisms can dominate the microflora in fresh minced fish and lightly preserved seafood at low temperatures. They are not often found in preserved seafood, since they are easily killed by the heat applied during

preservation. The Long & Hammer agar used in this experiment was not selective, and the count on the spread plates therefore includes all bacteria growing aerobically at the surface of this medium.

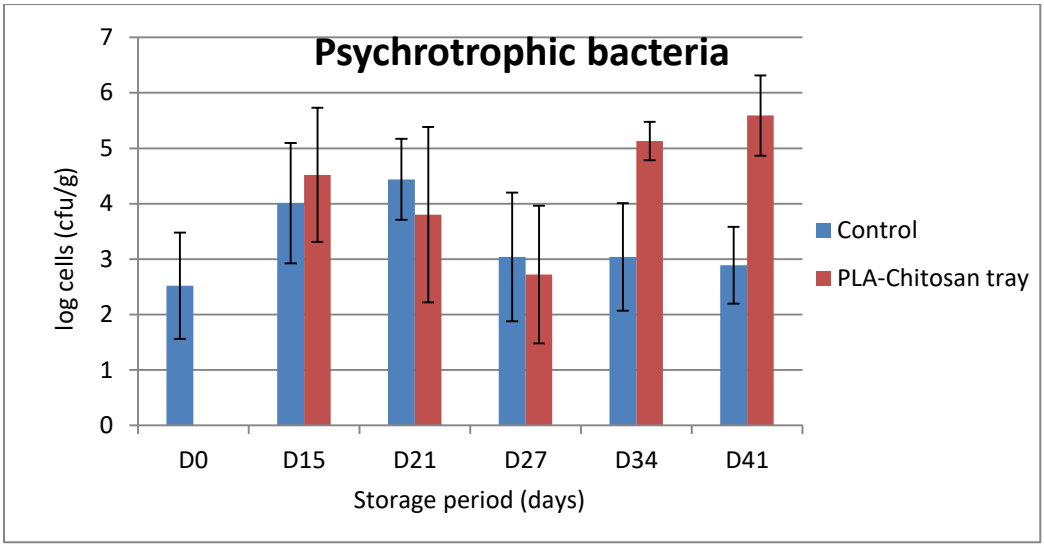

Figure 4.20: Psychrotrophic bacteria in log (cfu/g) on Long & Hammer medium. Sampling was performed in triplicate. The error bars represent standard deviation.

In the evaluation of the results of the packaging and storage experiment, it is important to consider the contact between chitosan and the bacteria on the salmon fillet. The salmon portion was only in contact with chitosan packaging material on one side. This side was tested specifically, and all samples were taken from a 0.5 cm layer in contact with the packaging material. Even with this focus on sampling procedure it was not detected increased antimicrobial effects of the chitosan packaging material. Chitosan is not volatile, and it does not affect bacteria that are not in direct contact with the compound. The kind of tray used in this study is commonly used for fresh or lightly preserved fish products, but lack the ability to have an activity on the non-contact sides. The antibacterial effect of the packaging material can be improved by using it for vacuum packaging, which would lead to all sides of the packaged food having contact with chitosan. However, this might not be enough as the bacteria move inside the food, and they can easily move away from where the chitosan is active.

A main observation was that the chitosan tray used had no antibacterial effect on the natural occurring bacteria on the smoked salmon. Similar observations have been found with sausages stored at 4°C for 14 days (García et al., 2010). The bacterial counts of their samples were equal for the control and chitosan-treated samples, similar to the results for the first 15 days of storage in this research. Other research groups have reported detectable antimicrobial effect of chitosan packaging on food matrices. Giatrakou, Ntzimani, & Savvaidis (2010) reported a shelf-life extension of 2 days for chicken samples that had been sprayed with a 1.5% chitosan solution, Bonilla et al. (2013) reported a significant antimicrobial activity of a PLA-film containing 10% chitosan tested on pork after incubation at 10°C for seven days, and Alak, Aras Hisar, Hisar, Kaban, & Kaya (2010) stated that chitosan caused a reduction in bacterial counts on fish fillet compared to vacuum and modified atmosphere packaging.

Although there have been reported many positive results from testing of chitosan in laboratory experiments, the results are mainly based on tests directly against the bacteria. When chitosan is applied in connection with a food matrix, there are many compounds that are not present in the

laboratory solutions, such as proteins, lipids, vitamins and others. These compounds might interact with the chitosan and possibly enhance or decrease its antibacterial effect (Alishahi & Aïder, 2012).

5 Conclusions

Several methods have been used to examine the effect of chitosan on *E. coli* and *S. aureus*. Limitations were found for all methods, but some general conclusions are found.

The contact method used, ISO 22196, showed that the best effect against *E. coli* was seen when the film contained MB as plasticizer, 8% polyethylene glycol and 2% modified chitosan with Cloisite 30B, giving a log reduction of 3.67 compared to the control. The best effect against *S. aureus* was seen from the film containing MB as plasticizer, polyethylene glycol and 1% of the rosehip-modified chitosan, resulting in a log reduction of 4.48.

Testing of chitosan film and powder in liquid bacteria solution gave results indicating that the best antibacterial effect of chitosan is found when the bacteria are under additional stress, e.g. chitosan and low incubation temperature (10°C). The results also showed that if the solution contained a high number of bacteria, the effect of chitosan could not be detected. This was most likely because only a low percent was in contact with the chitosan. The best effect was obtained when the bacteria were incubated together with chitosan powder at 10°C starting with a bacterial number about log 5 cfu/ml.

The growth curve analyses showed that the media that was used for the liquid bacteria solutions gave reliable growth curves with exponential growth. The TTD was linear related to the bacterial concentration at start of incubation, indicating that the media and incubation temperature used were suitable for both bacteria. An acid concentration of 0.5% almost completely dissolved the chitosan powder. *E. coli* was completely inhibited by this acid concentration without chitosan added, while *S. aureus* was able to grow. At this acid concentration with chitosan added, *S. aureus* never reached the detection limit, caused by the combination of low pH and chitosan. A concentration of acetic acid of 0.1% partially dissolved the chitosan powder, and gave a pH in the test solution that supported growth of both test bacteria. The chitosan colloid prepared with this acid concentration caused *E. coli* to reach the detection limit more than 8h after the control samples, and *S. aureus* more than 1h after the control samples. Both the growth rate and the maximum OD was affected, showing that both bacteria were inhibited by chitosan colloid prepared with acetic acid.

For the experiment with smoked salmon as a food matrix, there was not detected an effect of the chitosan-containing packaging tray. The chitosan tray used contained only 1% chitosan, which was unevenly distributed in the trays. The results of this tests show that it is important to test potential packaging materials on food matrices before commercial use, because in the food there is many more components that affect the bacteria and also the active components of the packaging films than there is in other laboratory experiments.

6 Future work

For the future work, it would be interesting to test the chitosan films on other spoilage and pathogenic bacteria in lab-scale experiments. The present work has shown that test methods vary in accuracy and what they can document for relevant packaging conditions. Supplementary test methods would therefore be valuable and needed to expand the knowledge on antimicrobial effects of chitosan. Chitosan used as colloid in mixtures can be effective due to large contact potential. Therefore, it would be interesting to examine the effect of other concentrations of both acetic acid and chitosan, and to detect which combinations are most effective.

In order to have the chitosan more evenly distributed in the films, colloids should be tested in the forming of chitosan films. This could lead to more even distribution of chitosan, compared to chitosan particles, and the results could be more predictable as all test films would have the same composition.

Future work should include testing the films on other food matrices, or in more complex laboratory solutions, to see if that give detectable effects. Also testing of films containing more chitosan together with other components could be of importance, as the components of the film might affect the antibacterial effect of chitosan.

The hurdle effect has been shown in the present work. Therefore, an alternative to films with only chitosan as active component could be films with addition of active volatile components such as essential oils. The active components could be used to make a coating solution that was applied inside the packaging tray to ensure contact between the food and active components such as chitosan.

7 References

Abdollahi, M., Rezaei, M., & Farzi, G. (2012). Improvement of active chitosan film properties with rosemary essential oil for food packaging. *International Journal of Food Science and Technology*, *47*(4), 847–853. http://doi.org/10.1111/j.1365-2621.2011.02917.x

Al-Sagheer, F. A., Ibrahim, E. I., & Khalil, K. D. (2014). Crystallinity, antimicrobial activity and dyeing properties of chitosan-g-poly(N-acryloyl morpholine) copolymer. *European Polymer Journal*, *58*, 164–172. http://doi.org/10.1016/j.eurpolymj.2014.06.027

Alak, G., Aras Hisar, S., Hisar, O., Kaban, G., & Kaya, M. (2010). Microbiological and Chemical Properties of Bonito Fish (*Sarda sarda*) Fillets Packaged with Chitosan Film, Modified Atmosphere and Vacuum. *Kafkas Universitesi Veteriner 'Fakultesi Dergisi*, *16*, S73–S80. http://doi.org/10.9775/kvfd.2009.1475

Albertos, I., Rico, D., Diez, A. M., González-Arnáiz, L., García-Casas, M. J., & Jaime, I. (2015). Effect of edible chitosan/clove oil films and high-pressure processing on the microbiological shelf life of trout fillets. *Journal of the Science of Food and Agriculture*, *95*(14), 2858–2865. http://doi.org/10.1002/jsfa.7026

Alboofetileh, M., Rezaei, M., Hosseini, H., & Abdollahi, M. (2014). Antimicrobial activity of alginate/clay nanocomposite films enriched with essential oils against three common foodborne pathogens. *Food Control*, *36*(1), 1–7. http://doi.org/10.1016/j.foodcont.2013.07.037

Alishahi, A., & Aïder, M. (2012). Applications of Chitosan in the Seafood Industry and Aquaculture: A Review. *Food and Bioprocess Technology*, 5(3), 817-830. http://doi.org/10.1007/s11947-011-0664-x

Appendini, P., & Hotchkiss, J. H. (2002). Review of antimicrobial food packaging. *Innovative Food Science and Emerging Technologies*, *3*, 113–126. http://doi.org/10.1016/S1466-8564(02)00012-7

Arancibia, M. Y., Alemán, A., Calvo, M. M., López-Caballero, M. E., Montero, P., & Gómez-Guillén, M. C. (2014). Antimicrobial and antioxidant chitosan solutions enriched with active shrimp (*Litopenaeus vannamei*) waste materials. *Food Hydrocolloids*, *35*, 710–717. http://doi.org/10.1016/j.foodhyd.2013.08.026

Arancibia, M. Y., Alemán, A., López-Caballero, M. E., Gómez-Guillén, M. C., & Montero, P. (2015). Development of active films of chitosan isolated by mild extraction with added protein concentrate from shrimp waste. *Food Hydrocolloids*, *43*, 91–99. http://doi.org/10.1016/j.foodhyd.2014.05.006

Baker, C. A., Rubinelli, P. M., Park, S. H., Carbonero, F., & Ricke, S. C. (2016). Shiga toxin-producing *Escherichia coli* in food: Incidence, ecology, and detection strategies. *Food Control*, *59*, 407–419. http://doi.org/10.1016/j.foodcont.2015.06.011

Baptista, I., Queirós, R. P., Cunha, Â., Rocha, S. M., Saraiva, J. A., & Almeida, A. (2015). Evaluation of resistance development and viability recovery by toxigenic and non-toxigenic *Staphylococcus aureus* strains after repeated cycles of high hydrostatic pressure. *Food Microbiology*, *46*, 515–520. http://doi.org/10.1016/j.fm.2014.09.016

Bonilla, J., Fortunati, E., Vargas, M., Chiralt, A., & Kenny, J. M. (2013). Effects of chitosan on the physicochemical and antimicrobial properties of PLA films. *Journal of Food Engineering*, *119*(2), 236–243. http://doi.org/10.1016/j.jfoodeng.2013.05.026

Chariyachotilert, C., Joshi, S., Selke, S. E. M., & Auras, R. (2012). Assessment of the properties of poly(L-lactic acid) sheets produced with differing amounts of postconsumer recycled poly(L-lactic acid). *Journal of Plastic Film and Sheeting*, *28*(4), 314–335. http://doi.org/10.1177/8756087911434337

Darie, R. N., Pâslaru, E., Sdrobis, A., Pricope, G. M., Hitruc, G. E., Poiata, A., … Vasile, C. (2014). Effect of Nanoclay Hydrophilicity on the Poly(lactic acid)/Clay Nanocomposites Properties. *Industrial & Engineering Chemistry Research*, *53*, 7877–7890. http://doi.org/10.1021/ie500577m

Day, B. P. F. (2008). Active Packaging of Food. In J. Kerry & P. Butler (Eds.), *Smart packaging technologies for fast moving consumer goods* (3rd ed., pp. 1–18). Portland, United States: John Wiley & Sons, Ltd. http://doi.org/10.1002/9780470753699

Devlieghere, F., Vermeulen, A., & Debevere, J. (2004). Chitosan: Antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables. *Food Microbiology*, *21*, 703–714. http://doi.org/10.1016/j.fm.2004.02.008

Dutta, J., Tripathi, S., & Dutta, P. K. (2012). Progress in antimicrobial activities of chitin, chitosan and its oligosaccharides: a systematic study needs for food applications. *Food Science and Technology International*, *18*(1), 3–34. http://doi.org/10.1177/1082013211399195

Fernandez-Saiz, P., Lagaron, J. M., & Ocio, M. J. (2009). Optimization of the biocide properties of chitosan for its application in the design of active films of interest in the food area. *Food Hydrocolloids*, *23*, 913–921. http://doi.org/10.1016/j.foodhyd.2008.06.001

Fetsch, A., Contzen, M., Hartelt, K., Kleiser, A., Maassen, S., Rau, J., … Strommenger, B. (2014). *Staphylococcus aureus* food-poisoning outbreak associated with the consumption of ice-cream. *International Journal of Food Microbiology*, *187*, 1–6. http://doi.org/10.1016/j.ijfoodmicro.2014.06.017

Food Safety: What you should know. (2015). Retrieved March 31, 2017, from http://www.searo.who.int/entity/world_health_day/2015/whd-what-you-should-know/en/

Forskrift om næringsmiddelhygiene. (2009). Retrieved June 9, 2017, from https://lovdata.no/dokument/SF/forskrift/2008-12-22-1623?q=forskrift om næringsmiddelhygiene

García, M., Díaz, R., Puerta, F., Beldarraín, T., González, J., & González, I. (2010). Influence of chitosan addition on quality properties of vacuum-packaged pork sausages. *Ciência E Tecnologia de Alimentos*, *30*(2), 560–564. http://doi.org/10.1590/S0101-20612010000200041

Giatrakou, V., Ntzimani, A., & Savvaidis, I. N. (2010). Effect of chitosan and thyme oil on a ready to cook chicken product. *Food Microbiology*, *27*(1), 132–136. http://doi.org/10.1016/j.fm.2009.09.005

Guirguis, O. W., Abd Elkader, M. F. H., & Nasrat, A. A. (2013). Enhancing antimicrobial activity for chitosan by adding Jojoba liquid wax. *Materials Letters*, *93*, 353–355. http://doi.org/10.1016/j.matlet.2012.11.096

Han, J. H., Patel, D., Kim, J. E., & Min, S. C. (2014). Retardation of *Listeria Monocytogenes* Growth in Mozzarella Cheese Using Antimicrobial Sachets Containing Rosemary Oil and Thyme Oil. *Journal of Food Science*, *79*(11), E2272–E2278. http://doi.org/10.1111/1750-3841.12659

Hobbs, G. (1982). Indicator organisms in fresh fish in relation to spoilage and public health. *Antonie van Leeuwenhoek*, *48*(6), 619–620. http://doi.org/10.1007/BF00399547

ISO 22196: Plastics - Measurement of antibacterial activity on plastics surfaces. (2007).

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). *Modern Food Microbiology*. (D. R. Heldman, Ed.) (7th ed.). Springer. http://doi.org/10.1007/b100840

Johnston, M. D. (1998). A simple and rapid test for quality control of liquid media, using the bioscreen microbiological growth analyser. *Journal of Microbiological Methods*, *32*, 37–43. http://doi.org/10.1016/S0167-7012(98)00007-4

Kong, M., Chen, X. G., Xing, K., & Park, H. J. (2010). Antimicrobial properties of chitosan and mode of action: A state of the art review. *International Journal of Food Microbiology*, *144*, 51–63. http://doi.org/10.1016/j.ijfoodmicro.2010.09.012

Koppell, J. (2011). International Organization for Standardization. In T. Hale & D. Held (Eds.), *Handbook of Transnational Governance* (pp. 289–295).

Leceta, I., Guerrero, P., & De La Caba, K. (2013). Functional properties of chitosan-based films. *Carbohydrate Polymers*, *93*(1), 339–346. http://doi.org/10.1016/j.carbpol.2012.04.031

Leceta, I., Guerrero, P., Ibarburu, I., Dueñas, M. T., & De La Caba, K. (2013). Characterization and antimicrobial analysis of chitosan-based films. *Journal of Food Engineering*, *116*, 889–899. http://doi.org/10.1016/j.jfoodeng.2013.01.022

Lee, J.-Y., Kim, S.-S., & Kang, D.-H. (2015). Effect of pH for inactivation of *Escherichia coli* O157:H7, *Salmonella Typhimurium* and *Listeria monocytogenes* in orange juice by ohmic heating. *LWT - Food Science and Technology*, *62*, 83–88. http://doi.org/10.1016/j.lwt.2015.01.020

Li, W., Coffin, D. R., Jin, T. Z., Latona, N., Liu, C. K., Liu, B., … Liu, L. (2012). Biodegradable composites from polyester and sugar beet pulp with antimicrobial coating for food packaging. *Journal of Applied Polymer Science*, *126*, E362–E373. http://doi.org/10.1002/app.36885

Liu, F., Qin, B., He, L., & Song, R. (2009). Novel starch/chitosan blending membrane: Antibacterial, permeable and mechanical properties. *Carbohydrate Polymers*, *78*(1), 146–150. http://doi.org/10.1016/j.carbpol.2009.03.021

Lucera, A., Costa, C., Conte, A., & Del Nobile, M. A. (2012). Food applications of natural antimicrobial compounds. *Frontiers in Microbiology*, *3*, 287. http://doi.org/10.3389/fmicb.2012.00287

Manni, L., Ghorbel-Bellaaj, O., Jellouli, K., Younes, I., & Nasri, M. (2010). Extraction and characterization of chitin, chitosan, and protein hydrolysates prepared from shrimp waste by treatment with crude protease from *Bacillus cereus* SV1. *Applied Biochemistry and Biotechnology*, *162*(2), 345–357. http://doi.org/10.1007/s12010-009-8846-y

Martínez-Camacho, A. P., Cortez-Rocha, M. O., Ezquerra-Brauer, J. M., Graciano-Verdugo, A. Z., Rodriguez-Félix, F., Castillo-Ortega, M. M., … Plascencia-Jatomea, M. (2010). Chitosan composite films: Thermal, structural, mechanical and antifungal properties. *Carbohydrate Polymers*, *82*(2), 305–315. http://doi.org/10.1016/j.carbpol.2010.04.069

Massouda, D. F., Visioli, D., Green, D. A., & Joerger, R. D. (2012). Extruded blends of chitosan and ethylene copolymers for antimicrobial packaging. *Packaging Technology and Science*, *25*, 321–327. http://doi.org/10.1002/pts

MicrobankTM Worldwide Performance Portfolio. (2011). Retrieved September 29, 2016, from http://www.pro-lab-direct.com/v/vspfiles/microbank/microbank-wwp-portfolio.pdf

Nan, L., Yang, K., & Ren, G. (2015). Anti-biofilm formation of a novel stainless steel against *Staphylococcus aureus*. *Materials Science & Engineering C*, *51*, 356–361. http://doi.org/10.1016/j.msec.2015.03.012

Nielsen, L. (2006). NMKL-184: Aerobic count and specific spoilage organisms in fish and fish products. Nordic Committee on Food Analysis.

No, H. K., Meyers, S. P., Prinyawiwatkul, W., & Xu, Z. (2007). Applications of chitosan for improvement of quality and shelf life of foods: A review. *Journal of Food Science*, *72*(5). http://doi.org/10.1111/j.1750-3841.2007.00383.x

Ouattara, B., Simard, R. E., Piette, G., Bégin, A., & Holley, R. A. (2000). Inhibition of surface spoilage bacteria in processed meats by application of antimicrobial films prepared with chitosan. *International Journal of Food Microbiology*, *62*(1–2), 139–148. http://doi.org/10.1016/S0168-1605(00)00407-4

Pariz Maluta, R., Morris Fairbrother, J., Eurides Stella, A., Cid Rigobelo, E., Martinez, R., & Antonio de vila, F. (2014). Potentially pathogenic *Escherichia coli* in healthy, pasture-raised sheep on farms and at the abattoir in Brazil. *Veterinary Microbiology*, *169*, 89–95. http://doi.org/10.1016/j.vetmic.2013.12.013

Portes, E., Gardrat, C., Castellan, A., & Coma, V. (2009). Environmentally friendly films based on chitosan and tetrahydrocurcuminoid derivatives exhibiting antibacterial and antioxidative properties. *Carbohydrate Polymers*, *76*(4), 578–584. http://doi.org/10.1016/j.carbpol.2008.11.031

Rabea, E. I., Badawy, M. E. T., Stevens, C. V., Smagghe, G., & Steurbaut, W. (2003). Chitosan as antimicrobial agent: Applications and mode of action. *Biomacromolecules*, *4*(6), 1457–1465. http://doi.org/10.1021/bm034130m

Randazzo, W., Jiménez-Belenguer, A., Settanni, L., Perdones, A., Moschetti, M., Palazzolo, E., … Moschetti, G. (2016). Antilisterial effect of citrus essential oils and their performance in edible film formulations. *Food Control*, *59*, 750–758. http://doi.org/10.1016/j.foodcont.2015.06.057

Râpă, M., Miteluţ, A. C., Tănase, E. E., Grosu, E., Popescu, P., Popa, M. E., … Vasile, C. (2016). Influence of chitosan on mechanical, thermal, barrier and antimicrobial properties of PLA-biocomposites for food packaging. *Composites Part B: Engineering*, *102*, 112–121. http://doi.org/10.1016/j.compositesb.2016.07.016

Realini, C. E., & Marcos, B. (2014). Active and intelligent packaging systems for a modern society. *Meat Science*, *98*, 404–419. http://doi.org/10.1016/j.meatsci.2014.06.031

Remya, S., Mohan, C. O., Bindu, J., Sivaraman, G. K., Venkateshwarlu, G., & Ravishankar, C. N. (2016). Effect of chitosan based active packaging film on the keeping quality of chilled stored barracuda fish. *Journal of Food Science and Technology*, *53*, 685–693. http://doi.org/10.1007/s13197-015-2018-6

Rhim, J. W., Hong, S. I., & Ha, C. S. (2009). Tensile, water vapor barrier and antimicrobial properties of PLA/nanoclay composite films. *LWT - Food Science and Technology*, *42*(2), 612–617. http://doi.org/10.1016/j.lwt.2008.02.015

Rhim, J. W., Hong, S. I., Park, H. M., & Ng, P. K. W. (2006). Preparation and Characterization of Chitosan-Based Nanocomposite Films with Antimicrobial Activity. *Journal of Agricultural and Food Chemistry*, *54*(16), 5814–5822. http://doi.org/10.1021/jf060658h

Sánchez-González, L., Cháfer, M., Hernández, M., Chiralt, A., & González-Martínez, C. (2011). Antimicrobial activity of polysaccharide films containing essential oils. *Food Control*, *22*(8), 1302–1310. http://doi.org/10.1016/j.foodcont.2011.02.004

Sébastien, F., Stéphane, G., Copinet, A., & Coma, V. (2006). Novel biodegradable films made from chitosan and poly(lactic acid) with antifungal properties against mycotoxinogen strains. *Carbohydrate Polymers*, *65*(2), 185–193. http://doi.org/10.1016/j.carbpol.2006.01.006

Shi, C., Zhao, X., Yan, H., Meng, R., Zhang, Y., Li, W., … Guo, N. (2016). Effect of tea tree oil on *Staphylococcus aureus* growth and enterotoxin production. *Food Control*, *62*, 257–263. http://doi.org/10.1016/j.foodcont.2015.10.049

Tassou, C. C., & Nychas, G. J. E. (1995). Antimicrobial activity of the essential oil of mastic gum (*Pistacia lentiscus* var. *chia*) on Gram positive and Gram negative bacteria in broth and in Model Food System. *International Biodeterioration and Biodegradation*, *36*(3–4), 411–420. http://doi.org/10.1016/0964-8305(95)00103-4

Torlak, E., & Sert, D. (2013). Antibacterial effectiveness of chitosan-propolis coated polypropylene films against foodborne pathogens. *International Journal of Biological Macromolecules*, *60*, 52–55. http://doi.org/10.1016/j.ijbiomac.2013.05.013

Trudeau, K., Vu, K. D., Shareck, F.-O., & Lacroix, M. (2012). Effect of gamma irradiation on the expressed proteins in the foodborne pathogen *Staphylococcus aureus*. *Radiation Physics and Chemistry2*, *81*, 1181–1184. http://doi.org/10.1016/j.radphyschem.2012.02.034

Turalija, M., Bischof, S., Budimir, A., & Gaan, S. (2016). Antimicrobial PLA films from environment friendly additives. *Composites Part B*, *102*, 94–99. http://doi.org/10.1016/j.compositesb.2016.07.017

Vardaka, V. D., Yehia, H. M., & Savvaidis, I. N. (2016). Effects of Citrox and chitosan on the survival of *Escherichia coli* O157: H7 and *Salmonella enterica* in vacuum-packaged turkey meat. *Food Microbiology*, *58*, 128–134. http://doi.org/10.1016/j.fm.2016.04.003

Wang, G.-H. (1992). Inhibition and inactivation of five species of foodborne pathogens by chitosan. *Journal of Food Protection*, *55*(11), 916–919. http://doi.org/10.4315/0362-028X-55.11.916

Wang, L., Liu, F., Jiang, Y., Chai, Z., Li, P., Cheng, Y., … Leng, X. (2011). Synergistic antimicrobial activities of natural essential oils with chitosan films. *Journal of Agricultural and Food Chemistry*, *59*(23), 12411–12419. http://doi.org/10.1021/jf203165k

Wickramarachchi, P. A. S. R., Samaratunge, I. N., Kaushalya, C. H. T. C., Rasangika, W. P. M., & Paranagama, P. A. (2016). Activity of chitosan films enriched with the essential oil of *Alpinia malaccensis* rhizome against *S. aureus*, *E. coli* and *C. Musae*. *Journal of the Natural Science Foundation of Sri Lanka*, *44*(2), 129–135. http://doi.org/http://doi.org/10.4038/jnsfsr.v44i2.7993

Yılmaz Atay, H., & Çelik, E. (2017). Investigations of antibacterial activity of chitosan in the polymeric composite coatings. *Progress in Organic Coatings*, *102*, 194–200. http://doi.org/10.1016/j.porgcoat.2016.10.013

Youssef, A. M., El-Sayed, S. M., Salama, H. H., El-Sayed, H. S., & Dufresne, A. (2015). Evaluation of bionanocomposites as packaging material on properties of soft white cheese during storage period. *Carbohydrate Polymers*, *132*, 274–285. http://doi.org/10.1016/j.carbpol.2015.06.075

Zhang, Y., Liu, X., Wang, Y., Jiang, P., & Quek, S. (2016). Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. *Food Control*, *59*, 282–289. http://doi.org/10.1016/j.foodcont.2015.05.032

Zivanovic, S., Chi, S., & Draughon, A. F. (2005). Antimicrobial Activity of Chitosan Films Enriched with Essential Oils. *Journal of Food Microbiology and Safety*, *70*(1), 45–51. http://doi.org/10.1111/j.1365-2621.2005.tb09045.x

Appendix

Appendix A – Experimental values

A-1 Antibacterial activity on plastic surfaces

Table A.1: Test of stomacher bag and PLA/ATBC/CS3 against *E. coli* and *S. aureus* by ISO 22196. All sampling were performed in triplicate.

Bacterium	Material	Incubation time [h]	log(cells/cm^2)	Standard deviation
E. coli	Stomacher bag	0	3.95	0.04
	Stomacher bag	24	4.78	0.94
	PLA/ATBC/CS3	24	2.37	0.19
S. aureus	Stomacher bag	0	3.54	0.08
	Stomacher bag	24	3.23	0.16
	PLA/ATBC/CS3	24	-0.34	0.24

Table A.2: Test of different test films against *E. coli* by ISO 22196. All sampling were performed in triplicate.

Material	Incubation time [h]	Log(cells/cm^2)	Standard deviation
Stomacher bag	0	3.78	0.03
Stomacher bag	24	5.94	0.11
PLA/MB/PEG8%/CS+C30B1%	24	0.72	0.93
PLA/MB/PEG8%/CS+C30B2%	24	0.11	0.87
PLA/MB/PEG8%/CS+C30B3%	24	0.71	0.57
PLA/MB/CS-M1%/BYK1%	24	1.52	1.04
PLA/MB/CS-M1%/BYK3%	24	1.20	0.68

Table A.3: Test of different test films against *S. aureus* by ISO 22196. All sampling were performed in triplicate.

Material	Incubation time [h]	Log(cells/cm^2)	Standard deviation
Stomacher bag	0	4.24	0.02
Stomacher bag	24	0.00	0.00
PLA/MB/PEG8%	24	-0.14	0.26
PLA/MB/PEG8%/CS-M1%	24	-0.24	0.21
PLA/MB/PEG6%/CS-M1%	24	-0.24	0.21
PLA/MB/PEG6%/CS+C30B1%	24	-0.07	0.10
Stomacher bag	0	4.39	0.04
Stomacher bag	24	1.41	0.42
PLA/MB/CS-M1%	24	1.09	0.39
PLA/MB/CS1%/HPS3%	24	2.66	0.29
PLA/MB/CS1%/HPS3%/BYK3%	24	1.41	0.43

A-2 Test of chitosan film in liquid bacteria solution

Table A.4: Test of PLA/ATBC/CS5 against *E. coli* and *S. aureus* in liquid bacteria solution at 30°C. All sampling were performed in triplicate.

Start concentration	Bacterium	Incubation time [h]	Film amount	Log(cfu/ml)	Standard deviation
High	*E. coli*	0	0	7.54	-
		24	0	9.17	0.04
		24	10	9.16	0.01
		24	25	9.18	0.03
	S. aureus	0	0	7.35	-
		24	0	8.89	0.05
		24	10	8.69	0.06
		24	25	8.74	0.02
Low	*E. coli*	0	0	5.41	-
		24	0	7.26	0.01
		24	10	7.23	0.03
		24	25	7.18	0.12
	S. aureus	0	0	5.39	-
		24	0	7.16	0.04
		24	10	6.89	0.05
		24	25	3.90	0.14

Table A.5: Test of PLA/ATBC/CS5 against *E. coli* and *S. aureus* in liquid bacteria solution at 10°C, starting with a high concentration of bacteria. All sampling were performed in triplicate.

		E. coli		*S. aureus*	
Film amount [cm^2]	Incubation time [days]	log(cfu/ml)	Standard deviation	log(cfu/ml)	Standard deviation
0	0	7.61	-	8.20	-
0	2	7.86	0.07	7.31	0.02
	5	8.71	0.05	7.35	0.03
	7	8.57	0.05	6.98	0.12
	9	8.54	0.05	6.88	0.00
10	2	7.88	0.12	7.25	0.07
	5	8.76	0.10	7.35	0.01
	7	8.64	0.06	6.92	0.27
	9	8.60	0.07	6.90	0.03
25	2	7.79	0.23	7.34	0.05
	5	8.64	0.14	7.36	0.01
	7	8.54	0.06	6.96	0.10
	9	8.61	0.07	6.88	0.08

Table A.6: Test of PLA/ATBC/CS5 against *E. coli* and *S. aureus* in liquid bacteria solution at 10°C, starting with a low concentration of bacteria. All sampling were performed in triplicate.

		E. coli		*S. aureus*	
Film amount [cm^2]	Incubation time [days]	log(cfu/ml)	Standard deviation	log(cfu/ml)	Standard deviation
0	0	5.41	-	5.20	-
0	5	6.30	0.16	4.90	0.04
	7	6.38	0.12	4.56	0.02
	14	6.43	0.11	3.71	0.06
10	5	3.28	2.35	1.96	1.40
	7	3.42	2.42	ND	0.00
	14	3.27	2.57	ND	0.00
25	5	1.06	1.50	1.80	0.14
	7	ND	0.00	ND	0.00
	14	ND	0.00	ND	0.00

ND = not detectable; no colonies detected in neither of the parallel samples.

A-3 Test of chitosan powder in liquid bacteria solution

Table A.7: Test of chitosan powder against *E. coli* and *S. aureus* in liquid bacteria solution at 30°C, starting with a high concentration of bacteria. All sampling were performed in triplicate.

		E. coli		*S. aureus*	
mg chitosan	Incubation time [days]	log(cfu/ml)	Standard deviation	log(cfu/ml)	Standard deviation
0	0	7.49	-	7.41	-
0	2	9.04	0.18	8.96	0.05
	6	9.07	0.12	8.48	0.12
	8	9.08	0.02	8.29	0.08
	10	8.93	0.03	8.27	0.05
	14	9.39	0.24	8.05	0.03
8	2	9.03	0.01	8.64	0.14
	6	9.11	0.01	8.81	0.06
	8	9.06	0.07	8.37	0.16
	10	8.76	0.07	8.33	0.02
	14	8.45	0.69	8.12	0.15
20	2	9.12	0.12	8.65	0.04
	6	9.22	0.03	8.53	0.11
	8	9.13	0.09	7.83	0.27
	10	8.82	0.41	8.23	0.22
	14	8.08	0.08	8.12	0.36

Table A.8: Test of chitosan powder against *E. coli* and *S. aureus* in liquid bacteria solution at 30°C, starting with a low concentration of bacteria. All sampling were performed in triplicate.

		E. coli		*S. aureus*	
mg chitosan	Incubation time [days]	log(cfu/ml)	Standard deviation	log(cfu/ml)	Standard deviation
0	0	5.58	-	5.52	-
0	2			6.67	0.01
	6	7.21	0.02	5.06	0.26
	8	7.18	0.06	4.47	0.02
	10	7.21	0.02	4.20	0.05
	14	7.07	0.02	2.97	0.41
8	2				
	6	3.64	2.69	3.87	1.47
	8	3.46	2.56	1.88	0.28
	10	3.46	2.63	2.23	0.00
	14	3.36	2.56	3.18	1.85
20	2			3.49	0.40
	6	5.07	0.44	2.44	0.43
	8	4.51	0.29	0.80	1.13
	10	4.54	0.00	0.83	1.18
	14	4.90	0.47	ND	0.00

ND = not detectable; no colonies detected in neither of the parallel samples.
Grey filled cells: Number not available due to plating of wrong dilutions.

Table A.9: Test of chitosan powder against *E. coli* and *S. aureus* in liquid bacteria solution at 10°C, starting with a high concentration of bacteria. All sampling were performed in triplicate.

		E. coli		*S. aureus*	
mg chitosan	Incubation time [days]	log(cfu/ml)	Standard deviation	log(cfu/ml)	Standard deviation
0	0	7.53	-	7.47	-
0	2	8.20	0.09	7.58	0.05
	6	8.64	0.02	7.41	0.02
	8	8.56	0.08	7.22	0.05
	10	8.61	0.06	7.16	0.02
	14	8.45	0.15	6.95	0.04
8	2	6.12	0.24	7.57	0.09
	6	7.48	0.18	7.38	0.11
	8	7.90	0.22	7.13	0.15
	10	8.36	0.14	7.09	0.17
	14	8.46	0.06	7.01	0.13
20	2	5.82	0.04	7.49	0.21
	6	7.25	0.36	6.92	0.17
	8	7.74	0.35	7.02	0.07
	10	8.38	0.05	7.20	0.05
	14	8.61	0.04	7.34	0.12

Table A.10: Test of chitosan powder against *E. coli* and *S. aureus* in liquid bacteria solution at 10°C, starting with a low concentration of bacteria. All sampling were performed in triplicate.

		E. coli		*S. aureus*	
mg chitosan	Incubation time [days]	log(cfu/ml)	Standard deviation	log(cfu/ml)	Standard deviation
0	0	5.64	-	5.49	-
0	2	5.61	0.07	4.92	0.10
	6	5.61	0.15	4.58	0.04
	8	5.59	0.12	4.04	0.02
	10	5.63	0.10	3.79	0.02
	14	5.63	0.08	3.17	0.05
8	2	2.28	0.14	1.13	0.80
	6	ND	0.00	0.47	0.33
	8	ND	0.00	ND	0.00
	10	ND	0.00	ND	0.00
	14	ND	0.00	ND	0.00
20	2	2.22	0.10	1.59	1.13
	6	0.47	0.33	0.47	0.33
	8	ND	0.00	ND	0.00
	10	0.23	0.33	0.23	0.33
	14	0.23	0.33	ND	0.00

ND = not detectable; no colonies detected in neither of the parallel samples.

A-4 Experiments with food matrix

Table A.11: White colonies on Iron agar. All sampling were performed in triplicate.

Incubation time [days]	Control		Chitosan tray	
	log(cfu/g)	Standard deviation	log(cfu/g)	Standard deviation
0	1.20	0.71	-	-
15	3.25	0.28	3.22	0.25
21	3.88	0.31	3.11	1.29
27	2.11	1.22	2.87	1.50
34	3.26	0.56	5.08	0.32
41	3.39	0.64	5.05	0.32

Table A.12: Total viable count on Iron agar. All sampling were performed in triplicate.

Incubation time [days]	Control		Chitosan tray	
	log(cfu/g)	Standard deviation	log(cfu/g)	Standard deviation
0	1.40	0.57	-	-
15	3.25	0.28	3.22	0.25
21	3.88	0.31	3.13	1.28
27	2.22	1.11	2.93	1.42
34	3.27	0.56	5.08	0.32
41	3.39	0.64	5.05	0.32

Table A.13: Colonies on Long & Hammer agar. All sampling were performed in triplicate.

Incubation time [days]	Control		Chitosan tray	
	log(cfu/g)	Standard deviation	log(cfu/g)	Standard deviation
0	2.52	0.96	-	-
15	4.01	1.09	4.52	1.21
21	4.44	0.73	3.80	1.58
27	3.04	1.16	2.72	1.24
34	3.04	0.97	5.13	0.34
41	2.89	0.69	5.59	0.73

Appendix B – Data calculated from growth curves

B-1 Data used for calibration curves

Table B.1: Data for *E. coli* used to make calibration curve. Detection limit: OD_{600}=0.2.

Log cells [cfu/ml]	Formula used for calculation (y=OD_{600}, x=time)	Time to detection [hh:mm]
1	y = 2.7454x - 0.6305	07:15
2	y = 3.4904x - 0.7162	06:18
3	y = 3.3053x - 0.5176	05:13
4	y = 3.8181x - 0.4759	04:15
5	y = 3.7472x - 0.3031	03:13
6	y = 3.4017x - 0.1036	02:09
7	y = 4.0545x + 0.0531	00:52

Table B.2: Data for *S. aureus* used to make calibration curve. Detection limit: OD_{600}=0.2.

Log cells [cfu/ml]	Formula used for calculation (y=OD_{600}, x=time)	Time to detection [hh:mm]
1	y = 3.9328x - 1.4899	10:19
2	y = 4.3342x - 1.4227	08:59
3	y = 4.1745x - 1.0705	07:18
4	y = 4.5948x - 0.9022	05:45
5	y = 4.9727x - 0.6893	04:18
6	y = 5.4372x - 0.4277	02:46
7	y = 5.7904x - 0.0874	01:11

B-2 Time to detection

Table B.3: Detection times for the different solutions with 0.1% and 0.5% acetic acid. Detection limit: OD_{600}=0.1.

Bacterium	Acid concentration	Additive/Time to detection		
		Water	Acetic acid	Chitosan colloid
E. coli	0.1%	02:03	02:16	10:56
	0.5%	02:03	*	*
S. aureus	0.1%	03:00	03:16	04:19
	0.5%	03:00	07:04	*

*=sample never reached the detection limit