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EFFECT OF MOLECULAR WEIGHT REDUCTION BY GAMMA IRRADIATION ON THE ANTIMICROBIAL ACTIVITY OF CHITOSAN

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EFFECT OF MOLECULAR WEIGHT REDUCTION BY GAMMA IRRADIATION ON THE ANTIMICROBIAL ACTIVITY OF CHITOSAN

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by
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ABSTRACT

The antimicrobial effectiveness of lower molecular weight (LMW) chitosan solutions was tested against *Listeria innocua* inoculated on TSA and MOX agar plates by the direct droplet method. The chitosan solutions were prepared by dissolving chitosan in 1% (v/v) acetic acid at concentrations ranging from 0.5 to 2.0% and irradiating with Co^{60} gamma rays ranging from 1 to 20 kGy. MW measurements of chitosan were determined by using HPSEC-MALLS-RI. The MW of chitosan varied from 114.8 kDa to 17.4 kDa and decreased with increasing irradiation dosages. Plate counts on TSA agar from shaker flask assays showed no significant differences among chitosan solutions irradiated up to 10 kGy and non-irradiated chitosan solutions. The inhibition of *L. innocua* by these solutions was 4 logs CFU/ml. However, after 12 hours the mean total plate count for irradiated as well as non-irradiated chitosan increased continuously. Chitosan solutions irradiated over 10 kGy were found to be ineffective against *L. innocua*, indicating that chitosan loses antimicrobial properties at higher irradiation levels with corresponding MW below 22.4 kDa. Chitosan and LMW chitosans were coated to 2.62 mg/in² on corona treated Cryovac® HangPak™ film and examined for inhibition zones on direct contact with 10^5 CFU/ml inoculated *L. innocua* TSA agar plates. The results clearly showed neither inhibition zones nor diffusion for non irradiated as well as irradiated chitosan samples. The same results were obtained with paper disks dipped in chitosan and dried for 24 hours. The average amount of chitosan absorbed was found to be 26mg/disk. Native and LMW chitosan bound in the paper disk matrix and was not released into the

surrounding media. However, wet paper disks showed clear inhibition zones around the disks for native as well as LMW chitosan.

DEDICATION

I dedicate this work to my parents, Gülsüm and Ramadan Sirmats. Their love and support have made this accomplishment possible.

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INTRODUCTION

Research in the area of effective antimicrobial food packaging materials has significantly increased during the past decade. Consumer behaviour has changed from traditional packaging concepts to the demand for minimally processed, easily prepared and ready-to-eat, fresh food products with a long shelf life (Vermeiren et al., 1999). Together with these consumer demands, requirements to the food packaging industry has also changed as a result of globalized companies with central distribution, increased distribution distances and longer storage time and temperature requirements (Appendini and Hotchkiss, 2002). Recent food-borne microbial outbreaks are driving a search for innovative ways to inhibit microbial growth in foods while maintaining quality, freshness, and safety. Active food packaging systems have significantly impacted packaging by extending the shelf-life and improving safety while maintaining quality, freshness, and safety (Vermeiren et al., 1999). The package is interacting with the product or the headspace between the package and the food to obtain a desired outcome (Rooney, 1995).

Antimicrobial packaging is a form of active packaging. Antimicrobial food packaging acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packaged food or packaging material itself. The microbial contamination of most foods occurs primarily at the surface due to surface contamination. Antimicrobial polymer compounds such as chitosan can act by direct contact or undergo positive migration from the package structure into the foods to reach potential inner contamination (Lagaron et al., 2007). Chitosan has been approved for use as a food

additive in Japan and Korea since 1983 and 1995, respectively (KFDA, 1995), and thus considerable attention has been given to the use of chitosan as an antibacterial agent to improve shelf-life of foods (No et al., 2006). Chitin, chitosan and their derivates are currently not approved as food additives or packaging materials by the European Union and the USA, therefore its applications are limited (Technical Insights, 1998).

Chitin is the second most abundant natural biopolymer derived from the exoskeletons of crustaceans and insects and also from cell walls of fungi. Chitosan, which is derived from N-deacetylation of chitin in the presence of hot alkali, has antimicrobial properties due to its unique polycationic nature. Chitosan is soluble in aqueous acidic medium due to the presence of charged amino groups (Mahlous et al., 2007). Extensive studies have been completed on chitosan to test its potential applications in the pharmaceutical, cosmetical and food industries. Due to its versatile biocompatibility and complete biodegradability in combination with low toxicity, it is very important to exploit the unique properties and to realize the full potential of chitosan.

Low-molecular weight chitosan can be prepared by chemical, radiation, or enzymatic degradation of the high-molecular weight polymer. Radiation can provide a useful tool for degradation of different polymers. In the reaction, no other chemical reagents are introduced and there is not a need to control the temperature, environment or additives (Feng et al., 2008). It is reported that radiation can induce reactions such as chain scissions of the 1-4 glycosidic bonds which causes a reduction in molecular weight of the polymer and negligible cross-linking (Lim et al., 1997). Previous research proved

that lowering the molecular weight of chitosan increased antioxidant activity (Feng et al., 2008). However, research is necessary to determine the antimicrobial activity of chitosan as the molecular weight is reduced.

The primary objective of this research was to determine the influence of reducing the molecular weight of chitosan on its antimicrobial activity. Molecular weight reduction was achieved with increasing dosages of gamma irradiation. Another objective of this study was to determine if lower molecular weight chitosan desorbs from the surface of a solid matrix, such as paper or polymeric film, and diffuses into a surrounding medium in which it is in contact. This would determine the relative effectiveness of chitosan as an antimicrobial for use in active packaging.

LITERATURE REVIEW

Active Packaging

In the early 19th century Nicholas Appert discovered that airtight closed bottles, soaked in boiling water, could preserve foods for long periods of time. Scientific research in understanding the relationships between packaging, shelf life, safety and processing/storage conditions were born (Hotchkiss, 1995, Brody, 2007). Since then distribution practices associated with globalisation, new consumer product logistics, new distribution trends (internet shopping), automatic handling systems at distribution centers, and consumer health and safety have changed toward consumer preferences demanding for minimally processed food with fewer preservatives, higher nutritive value, and fresh sensory attributes.

As a result of these driving forces active packaging technologies are being developed. Active packaging is a form of dynamic packaging in which the package, the product and the environment interact to extend shelf-life or enhance safety or sensory properties while retaining the quality of the product. Active packaging systems include oxygen scavenging, control of moisture absorption, carbon dioxide and ethanol generation, and antimicrobial packaging with migrating and non-migrating systems (Suppakul et al., 2003, Vermeiren et al.. 1999, Suppakul et al., 2008).

Antimicrobials

Antimicrobials are compounds that have been developed to block, interfere, or suppress the growth of microbes that can cause illness and financial loss due to spoilage.

The major targets for antimicrobials are pathogens or food spoilage microorganisms whose metabolic end products cause off odors and flavours, texture problems, discoloration or slime (Suppakul et al. 2003). Consumers remain cautious about the chemical preservatives coming into their food chain (e.g. benzoates, nitrites, sulfites, sorbates, NaCl). For this reason, it is increasingly more important to use naturally derived antimicrobials as they represent a perceived lower risk to the consumer.

Using antimicrobial agents in "smart" packaging films should lead to increase food safety and shelf-life of perishable foods. Many antimicrobials function by disrupting the cell wall, cell membrane, metabolic enzymes, protein systems and genetic systems of the packaged foods (Nicholson 1998). The exact mechanisms are often unknown. The effectiveness of antimicrobials depends on the characteristics of the food product, its initial microbial load, water-activity, pH, storage and distribution conditions, and the target microorganisms.

Microbial factors that affect the antimicrobial activity include inherent resistance, initial number and growth rate of the organism, cellular composition and cellular injury. Plant-derived antimicrobial agents include low molecular weight components from herbs, spices and essential oils. Compounds from microorganisms include bacteriocins like nisin. Animal-derived antimicrobials include enzymes, such as lysozyme, chitosan, and chitin.

Research and development in the application of these antimicrobials is increasing with the advancement of technology (Suppakul et al., 2003). In addition, many natural compounds are classified in the U.S. as GRAS (Generally Recognized as Safe) and also

have advantages for human health. In food preservation, antimicrobials can be applied or incorporated into the food as a food ingredient, sanitizing treatment of equipment, spray or dip treatment of the product or as an active packaging system.

New models for incorporation with novel carriers can provide improved capability and effectiveness. All of these techniques represent a significant trend away from direct food additives (Nicholson, 1998).

Antimicrobial Packaging

Recent microbial outbreaks in food are demanding a search for innovative methods and applications to inhibit microbial growth in foods while maintaining quality, freshness and safety (Cooksey, 2001, Appendini and Hotchkiss, 2002). It is important to use packaging to provide an increased margin of safety and quality. Food packaging may include materials with antimicrobial properties. These new packaging technologies could play a role in extending shelf life of food and reducing the risk from microbial growth. Antimicrobial packaging is a form of active packaging (Appendini and Hotchkiss, 2002). Active packaging interacts with the product or the head space between the package and the food system to reduce, inhibit or retard the growth of microorganisms that may be present in the packaged food or packaging material itself (Labuza and Breene, 1989, Rooney, 1995). Two types of antimicrobial packaging have found commercial success: Those that indirectly incorporate an antimicrobial agent into the packaging film and those that directly incorporate one (Cooksey, 2001).

Indirectly Incorporated Antimicrobial Agents.

The most successful commercial application of antimicrobial packaging has been sachets or pads enclosed in the package, either loose or attached to the interior package. The most common forms of these indirectly incorporated antimicrobial agents are oxygen and moisture absorbers and ethanol vapor generators. Generally these absorbers are used in packaging to prevent oxidation and water condensation but they have no direct antimicrobial action. Low water activity is important to limit the growth of bacteria and yeast. A reduction in oxygen indirectly inhibits the growth of aerobes, particularly molds (Rooney 1995, Appendini and Hotchkiss, 2002). Smith et al. (1995) showed that oxygen absorbers placed inside the headspace of a food package reduced the oxygen levels to < 0.01% within 1-4 days at room temperature. Furthermore, oxygen and moisture absorbers are commonly made with iron powder and ascorbic acid.

However, because the technology involved is used mainly for food preservation, the material used inside the sachet must meet certain criteria prior to approval by regulatory agencies. One of the most important criteria is that it must not produce toxic substances or offensive odors/gases (Harima, 1990). More recent oxygen sachets are so small that they only cover the package label (Smith et al., 1995).

Ethanol vapour generators contain absorbed or encapsulated ethanol in a carrier material and are enclosed in packaging films with selective permeabilities, which allow the slow or rapid release of ethanol vapor. When food is packed with a sachet of ethanol, moisture is absorbed from the food, and ethanol vapour is released from the encapsulation and permeates the package headspace.

These sachets are relatively small and only effective in products with a water activity < 0.92, such as bakery and dried fish products (Smith et al., 1995, Appendini and Hotchkiss, 2002). Rooney (2007) and Smith et al. (1995) report that ethanol as a vapor spray on bakery products was a successful commercial application which increased mold-free shelf life by 50 -250%.

Also, after heating the bakery products the ethanol vaporised. Generally ethanol vapour generators consists of food grade alcohol and water (55% and 10% by weight, respectively) absorbed on to silicon dioxide powder (35%) and contained in a sachet made of a laminate of paper/ethylene- vinyl acetate copolymer.

To mask the odor of ethanol some sachets contain traces of vanilla or other flavors (Appendini and Hotchkiss, 2002). Further developments in ethanol pads include using several incorporated antimicrobial compounds such as chlorine dioxide, silver salts, bacteriocins and ozone or natural organic spices such as rosemary, oregano, sage, or thyme (Sivertsvik, 2007).

Oxygen and moisture absorbers and ethanol vapor emitters have advantages and disadvantages. Emitters indirectly inhibit microbial growth, extending the product's shelf life and reducing the cost required for gas flushing equipment. Furthermore, emitters maintain product qualities without additives. They also eliminate the need for preservatives such as benzoic or sorbic acid to control yeast spoilage. Sachets can be conveniently removed from packages.

On the other hand these sachets or pads have limited applications. Many foods are oxygen and moisture sensitive. Odor, off flavor and off color can easily occur.

Furthermore, useage in liquids and liquid foods is not possible. Consumers are concerned about sachets inside the packages and possible misuse of sachets. The expensive cost of the sachets limits their use to products with higher profit margins (Smith et al., 1995, Rooney, 1995).

Directly Incorporated Antimicrobial Agents.

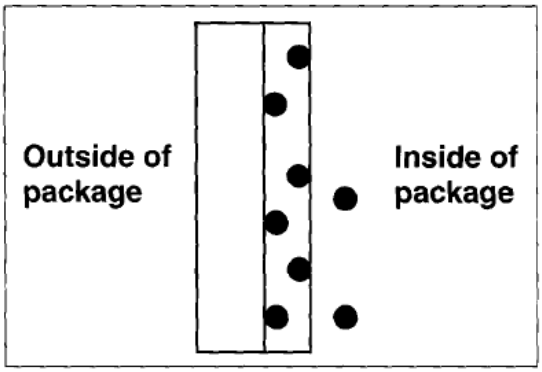
According to Cooksey (2001), Brody et al. (2001) and Rooney (1995) there are two main groups of antimicrobial films. The first group includes the direct incorporation of the antimicrobial agents into the packaging film. The antimicrobial agent can migrate to the surface of the package material and thus can contact food. The second group includes films coated with a material which acts as a carrier for the agent.

Both films are effective against food surface microbiological growth without migration of the active agent to the food. In both cases, direct contact with the packaged contents is necessary to be effective. Beside the two main groups there is also a subgroup which contains films composed of a polymer with antimicrobial characteristics. The antimicrobial additives in these polymer films must be able to resist the high temperatures which are required to melt and form the polymer films (Cooksey, 2001). Figure 1 shows a profile of a packaging film coated with antimicrobial agent and its release into the packaging contents.

The rate of the release would depend on the interaction between the antimicrobial agent, the coating material, the packaging content and the targeted bacteria. Figure 2 shows the migration of an antimicrobial agent from a film which has the antimicrobial

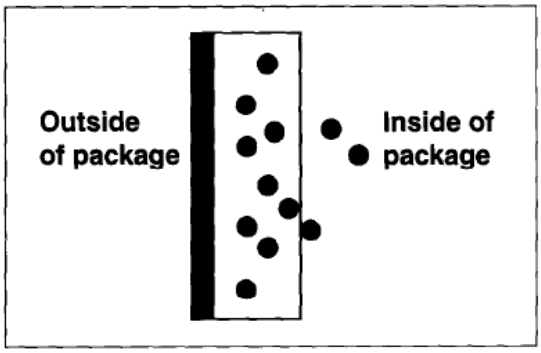
agent directly incorporated into the film. A barrier layer on the outside of the package might be necessary to prevent loss of the antimicrobial agent.

Figure 1. Profile of a Packaging Film Containing an Antimicrobial Coating



Source: (Cooksey 2001)

Figure 2. Profile of Polymer Film Containing Antimicrobial Agents with a Barrier Layer on the Outside Layer of the Packaging



Source: (Cooksey 2001)

Furthermore it is important that films and coatings are developed to allow a controlled and slow release of the antimicrobial additives into the packaged contents (Cooksey, 2001). Consumer concern about the use of synthetic compounds in the food chain has widened the interest in naturally occurring antimicrobial additives such as organic acids, bacteriocins, spice extracts, enzymes and essential oils (Nicholson, 1998).

Table 1 shows some examples of GRAS, non- GRAS and natural antimicrobial agents for potential use in food packaging materials.

Table 1. Examples of Antimicrobial Agents for Potential use in Food Packaging Materials

Antimicrobials	**Examples**
Organic Acids	Acetic, benzoic, citric, lactic, propionic, sorbic
Bacteriocins	Nisin, pediocin, lacticin
Enzymes	Peroxidase, lysozyme, chitinase, glucose oxidase
Essential oils (volatile plant extract)	Thymol, grapefruit seed, allyl isothiocyanate
Polysaccharide	Chitosan
Spice extracts	Cinnamic, caffeic acid, rosemary,
Metals	Silver, chopper
Fungicide	Imazalil, benomyl
Chelating agents	EDTA
Alcohol	Ethanol

Source : (Suppakul et al., 2008, Brody et al., 2001, Hotchkiss, 1995)

The most commonly used antimicrobial as a polymer additive is silver substituted zeolite, which has had a portion of its sodium ions replaced with silver ions. These substituted zeolites are thermostable (up to 800°C) and can be used for thermal processing methods such as extrusion and injection molding (Appendini and Hotchkiss, 2002). Therefore, they have been incorporated as a thin co-extruded layer with other polymers like polyethylene or polypropylene (Brody, 2001).

The purpose of the zeolite is the slow release of silver ions, with their microbial inhibition, into the food (Hotchkiss, 1995). The microbial cells absorb silver ions thereby

leading to the disruption of the enzymatic activity of the cells. Zeolites are commonly used in Japan and recently they received the approval of the FDA (Food and Drug administration) for use in food contact material. However, they have not been cleared by the European regulatory authorities (Suppakul et al., 2008).

Currently, no European regulation exists on active packaging. Components for food packages are required to belong to a positive list of approved compounds. The maximum migration limit from the packaging to the food was set at 60 mg/kg, which is incompatible with active packaging, especially when the packaging system is designed to release or absorb active ingredients of foods (Suppakul et al., 2008).

Antimicrobial agents have antimicrobial properties for certain kind of bacteria (gram-positive or gram-negative). Combinations of different antimicrobials incorporated into packaging films have successfully been investigated. For example, lysozyme effective against gram-positive bacteria in combination with chelating agents (i.e. EDTA) can also target gram negative bacteria. Addition of EDTA with nisin showed inhibitory effects on *E. coli* and *Salmonella typhimurium* (Appendini and Hotchkiss 2002).

Chitosan films made from dilute acetic acid solutions showed inhibition of *Rhodotorula ruba* and *Penecilium notatum* when directly applied into the colony. However, the interaction between the antimicrobial agent and the film-forming material may affect the casting process, the release of the antimicrobial agent and the mechanical properties of the film (Suppakul et al., 2008).

Begin and Calsteren (1999) showed that antimicrobial agents with a molecular weight larger than that of acetic acid can be used as multilayer films or for coating.

According to that study acetic acid diffused out of chitosan in an aqueous medium more rapidly than propionic acid (Ouattara et al., 2000). These results led to the suggestion that the release of organic acids from chitosan is a complex procedure that involves many factors such as electrostatic interactions, ionic osmosis, and structural changes in the polymer induced by the presence of the acids (Suppakul et al., 2008).

Apart from organic acids and polysaccharides, fungicides like imazalil and benomyl have been tested for antimicrobial activity in packaging. Imazalil and benomyl are effective when incorporated into LDPE for wrapping fruits and vegetables or preventing mold growth on cheese surfaces. LDPE film containing 1000 mg/kg imazalil substantially inhibited growth of *Penicillium* sp. (Weng and Hotchkiss, 1992). This work proved that antimycotic films could be effective for control of surface molds in food. Because of toxicological reasons imazalil and benomyl are not approved for food use (Hotchkiss, 1995). Weng and Hotchkiss (1993) reported that the incompatibility of organic, antimycotic acids such as propionic, benzoic, and sorbic acid with polymers such as LDPE is due to differences in polarity (nonpolar LDPE film and polar acid).

This problem was solved by forming the anhydride of the acid, which removed the ionized acid function and decreased polarity and created an active packaging component. Anhydrides are dry and thermally stable. When their activity is initiated by contacting with moisture in the food, hydrolysis takes place which leads to formation of free acids. The advantage of these free acids is that they migrate from the surface of the polymer film to the food, where they inhibit microbial growth (Weng and Hotchkiss, 1993). Volatile compounds and enzymes are heat sensitive antimicrobials.

To prevent heat denaturation of the enzyme, solvent compounding is used for their incorporation into polymers. Enzymes, such as lysozyme, have been successfully incorporated into cellulose ester films by solvent compounding (Appendini and Hotchkiss, 2002). Studies on the bacteriocin nisin, showed that the antimicrobial activity is higher when heat is not used in pressed films (Cha and Cooksey et al., 2003). Again, to achieve antimicrobial inhibition the release from the polymer film has to be maintained at a minimum rate so that the concentration of the antimicrobial agent on the foods surface is above a critical inhibitory concentration (Appendini and Hotchkiss, 2002).

Floros et al. (2000) achieved a constant controlled release to the food surface with the use of multilayer films such as a control layer/matrix layer/barrier layer. The control layer controls the release rate of the antimicrobial agent, the matrix layer contains the antimicrobial agent while the barrier layer prevents migration of the agent towards the outside of the package.

Non-volatile antimicrobials must contact the surface of the food to allow migration of the antimicrobial agents to the food. Volatile antimicrobials have the advantage that they do not necessarily require contact to the food surface, since they can diffuse throughout the food package (Appendini and Hotchkiss, 2002). The shelf life of fresh beef, cured pork, sliced raw tuna, cheese, egg sandwich, noodles, and pasta was enhanced when the package was flushed with allyl isothiocyanate (AIT) (Lim and Tung, 1997). These volatile compounds can be directly incorporated into the polymer or compounded, extruded or coated onto packaging films.

It has also been trapped in cyclodextrins and coated onto internal packaging labels. Chlorine dioxine, sulphur dioxide, carbon dioxide and AIT are volatile antimicrobials (Appendini and Hotchkiss, 2002). AIT is a plant derived component made out of black or brown mustard and is a very commonly used antimicrobial in Japan, but only when the compound is extracted from natural sources. AIT is currently not approved by the FDA for use in the U.S.A. due to a safety concern that this synthetic compound may be contaminated with traces of the toxic allyl chloride used in the manufacturing process (Suppakul et al., 2008).

According to Lim and Tung (1997) the antimicrobial effectiveness of AIT depends on the interaction with the packaging material. It was found that solubility, permeability and diffusion in polyvinylidene chloride (PVDC) and polyvinylchloride (PVC) copolymer films are dependant on temperature and concentration of AIT. One major drawback of a volatile antimicrobial is off flavour or odor. Furthermore, barrier materials need to be used to prevent permeation outwards from the package. External factors such as temperature, moisture and pressure could influence the shelf life of the package.

Coating of Antimicrobials on Polymer Surfaces.

The advantages of coated films are biodegradability, biocompatibility, edibility, and barrier properties against oxygen and physical interference. They can also serve as a carrier for antimicrobial agents (Brody et al., 2001). Coating and casting is useful for thermo-labile and difficult-to-process antimicrobials. Cooksey (2000) successfully coated LDPE film with nisin using methylcellulose/hydroxymethlycellulose as a carrier and

found that the coating suppressed *S. aureus* and *L. monocytogenes*. Manipulating the polymer structure can enhance antimicrobial adsorption.

The higher polarity of NaOH treated films enhanced the absorption of antimicrobials. To increase compatibility between the surfaces of polyoefins and bacteriocins, binders such as polyamide resins have been used (Appendini and Hotchkiss, 2002). Milk-based proteins can also serve as carriers for antimicrobial agents and preservatives (e.g. lysozyme, nisin, potassium sorbate, EDTA), as well as plasticizers (e.g. glycerine or sorbitol).

The charge density and the cavity size of proteins offer the potential to control the diffusion rate of incorporated antimicrobial agents using change of pH. Whey protein films impregnated with lysozyme maintained tensile strength at concentrations of up to 100 mg of lysozyme/g of dried film. The lysozyme slowly released from the film and showed a inhibition zone against *Brochoterix thermoshacta, a* spoilage microorganism (Brody et al., 2001).

Inherently Antimicrobial Polymers.

Inherently antimicrobial polymers such as chitosan have been used in films and coatings (Appendini and Hotchkiss, 2002). The cationic nature of chitosan which kills gram positive and gram negative bacteria, allows it to be used as an antimicrobial coating to protect fresh vegetables and fruits from degradation (Cuq et al., 1995). A study on strawberries coated with either 1% or 1.5% chitosan (CS) or chitosan combined with calcium gluconate (CaGlu) showed a positive application of chitosan.

The strawberries were stored at 10 °C for one week. The effectiveness of the treatments in extending fruit shelf-life was evaluated by determining fungal decay, respiration rate, quality attributes and overall visual appearance. No sign of fungal decay was observed during the storage period for fruit coated with 1.5% CS (with or without the addition of CaGlu) or 1% CS + 0.5% CaGlu. By contrast, 12.5% of the strawberries coated with 1% CS lacking calcium salt were infected after five days of storage. The chitosan coating reduced respiration activity, thus delaying ripening and the progress of fruit decay due to senescence.

Chitosan coatings delayed changes in weight loss, firmness and external color compared to untreated samples. Strawberries coated with 1.5% chitosan exhibited less weight loss and reduced darkening than those treated with 1% chitosan, independently of the presence or absence of CaGlu (Munoz et al., 2008). The chitosan may act as a barrier between the nutrients in the packaging content and the microorganisms. In addition, chitosan based antimicrobial films or coatings have been used to carry organic acids and spices (Ouattara et al., 2000, Bégin and Calsteren, 1999).

A recent study showed the efficacy of chitosan coated plastic films incorporating five Generally Recognized as Safe (GRAS) antimicrobials (nisin, sodium lactate (SL), sodium diacetate (SD), potassium sorbate (PS) and sodium benzoate (SB)) against *Listeria monocytogenes* on cold-smoked salmon. Salmon samples were surface-inoculated with a five-strain cocktail of *L. monocytogenes* and packaged in chitosan coated plastic films containing 500 IU/cm^2 of nisin and 9 mg/cm^2 of SL, 0.5 mg/cm^2 of

SD, 0.6 mg/cm^2 of PS, or 0.2 mg/cm^2 of SB, and stored at room temperature (ca. 20 °C) for 10 days.

The film incorporating SL was the most effective, completely inhibiting the growth of *L. monocytogenes* during 10 days of storage. *L. monocytogenes* in samples packaged in the other four antimicrobial films grew, but the increase in counts was lower than the control. Among all the treatments, chitosan coated plastic films with 4.5 mg/cm^2 SL, 4.5 mg/cm^2 SL–0.6 mg/cm^2 PS and 2.3 mg/cm^2 SL–500 IU/cm^2 nisin were the most effective. These three most effective antimicrobial films were then tested at refrigerated temperature. They completely inhibited the growth of *L. monocytogenes* on smoked salmon for at least 6 weeks. Chitosan coated plastic films containing 4.5 mg/cm^2 SL can potentially assist the smoked-salmon processing industry in their efforts to control *L. monocytogenes* (Ye et al., 2008).

Chitin and Chitosan

Chitin is the second most abundant polymer after cellulose. It is the main component in crustacean shells, insect exoskeletons, and fungal cell walls (Vanson, 1995 and Muzarelli, 1977). Crustacean shell waste is composed of chitin (15-30%), protein (15-40%), and calcium carbonate (35-55%) (Johnson and Peniston, 1982). Chitin is crystalline and insoluble in ordinary solvents in its native state (Muzarelli, 1977). Chitin has three polymorphic structures known as alpha, beta and gamma. The alpha-chitin is the dominant and most stable form and is usually found in crustaceans, insects and fungi. Alpha-chitin has an antiparallel chain structure, while beta-chitin obtained from the pen of the squid has a parallel chain structure. The antiparallel chain or sheet arrangement of

alpha-chitin induces hydrogen bonding (Blackwell, 1969). These two forms of chitin show different swelling behaviours. Alpha-chitin is usually insoluble in all common organic solvents with the exception of dimetlyacetamide with lithium chloride while beta-chitin swells in water and dissolves in formic acid. In contrast to these compounds, gamma-chitin has been poorly explored (Blackwell, 1969). Figure 3 shows the recovery of chitosan from chitin and shellfish waste, which involves different chemical processes.

Commercial chitin products are usually prepared from the shells of crab and shrimp by treatment with dilute NaOH solution for deproteinization, followed by treatment with a dilute HCL solution demineralization. Chitosan is the deacetylated form of chitin and is the most important derivative of chitin due to its solubility in dilute acids (Figure 3). Commercial chitosan is produced by deacetylation of chitin using NaOH solution at high temperatures (Muzarelli, 1977). Chemically, chitin is a ß-(1-4)-linked glycan composed of glucosamine and N-acetylated glucosamine (2-acetamino-2-deoxy-D-glucose) units linked by glycosidic bonds, while chitosan is composed primarily of 2-amino-2-deoxy-D-glucose (glucosamine) (Figure 4).

Glucosamine and its N-acetylated from are the most abundant amino sugars occurring in polysaccharides, glycoproteins, and cell walls (Blackwell, 1969). Figure 4 shows structural differences of chitin, chitosan and cellulose. The primary difference among these compounds is seen in the C-2 position. Chitin has acetamide groups, $NHCOCH_3$, whereas chitosan has amine groups, NH_2, and cellulose has a hydroxyl group (OH) (Park, 2001). Sandford (1989) stated that chitosan is a linear polyamide whose amino groups can be used in chemical reaction and the primary (C6) and secondary (C3)

hydroxyl groups are available for derivitization. However, acetamide groups in chitin or amine groups in chitosan give it significantly different properties than cellulose (Hon, 1994). The degree of deactelyation of commercial chitosan products vary from 60 to 98%. Average molecular weights of chitosan largely depend on their origins and isolation processes (Muzarelli, 1977).

The three types of reactive functional groups of chitosan are the amino group, as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions (Furusaki et al., 1996). These polysaccharides are renewable resources which are currently being explored intensively for their applications in pharmaceutical, cosmetic, biomedical, biotechnological, agricultural, food, and non-food industries (Gupta and Ravikumar, 2000). Furthermore, chitosan with free amine groups ($-NH_2$) is insoluble in water. Because of its ability to form salts, an amine that is insoluble in water can be made soluble by treatment with dilute carboxylic acid such as acetic acid.

The presence of amine groups in chitosan facilitates bringing the polymer into solution as a result of the formation of $-NH_3^+$ (Park, 2001). The neutralized form of chitosan reacts with a variety of metals such as copper, chromium, cadmium, manganese, cobalt, lead, mercury, zinc, uranium, palladium, and silver (Sandford and Hutchings, 1987). Chitosan can be used as a new raw material to make biodegradable polymer films. Chitin and chitosan are nontoxic, non allergenic and free from pyrogens, so the body is not likely to reject these compound as foreign invaders (Skjak et al., 1988, Kanatt et al., 2008). Even though chitosan is non-toxic, the use of this natural material in foods has been limited by regulatory considerations in the United States.

Chitin, chitosan and their derivatives are currently not approved for food additives or packaging materials in the United States (Technical Insights, 1998). Japan has considerably fewer restrictions on chitosan uses compared with the United States. Chitosan has been approved for use as a food additive in Japan and Korea since 1983 and 1995, respectively (KFDA, 1995), and thus considerable attention has been given to the use of chitosan as an antibacterial agent to improve shelf-life of foods (No et al., 2006).

Figure 3. Detailed Flowchart for Chitin and Chitosan Preparation

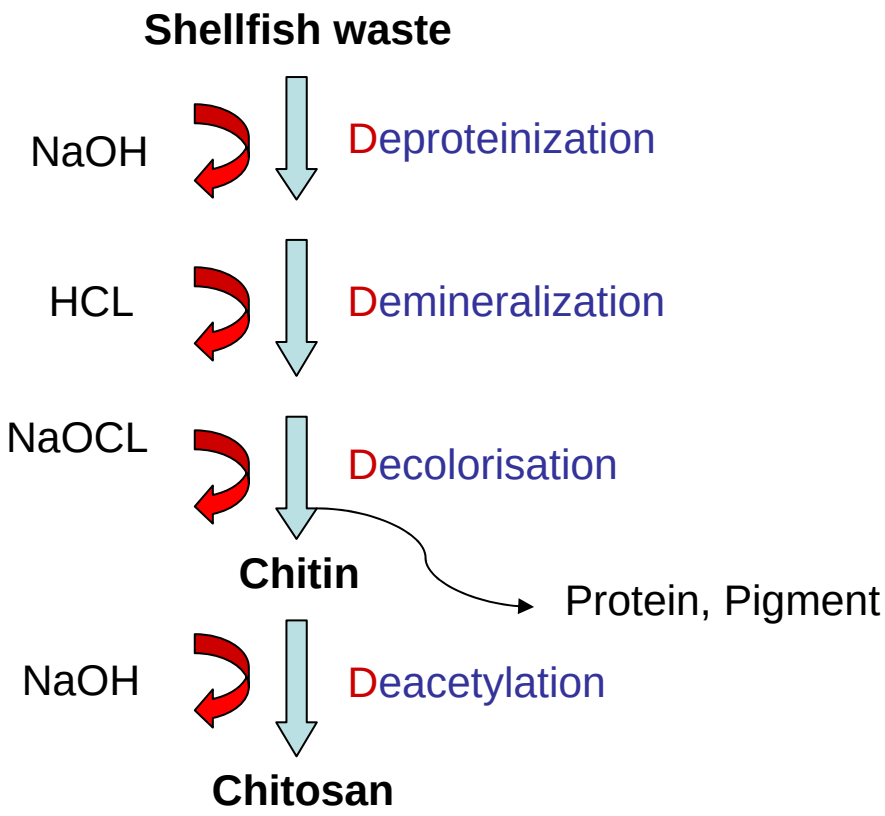
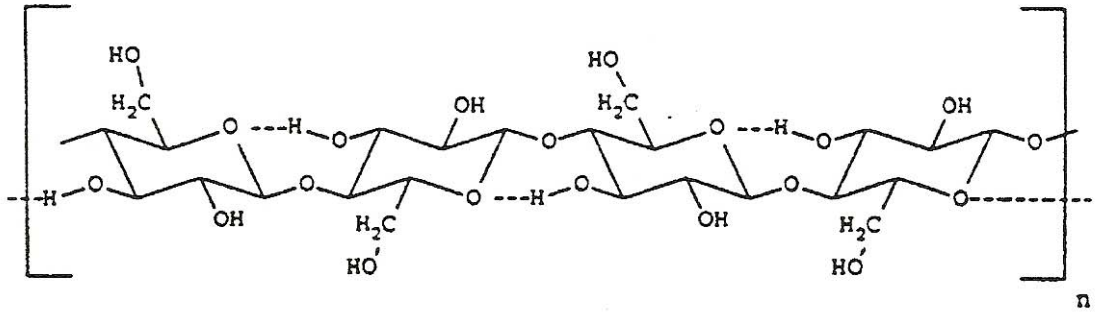
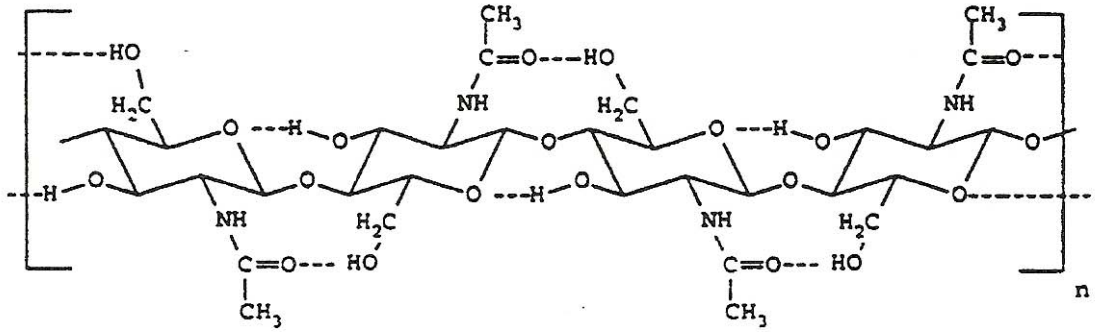


Figure 4. Structural Similarities of Chitin, Chitosan and Cellulose

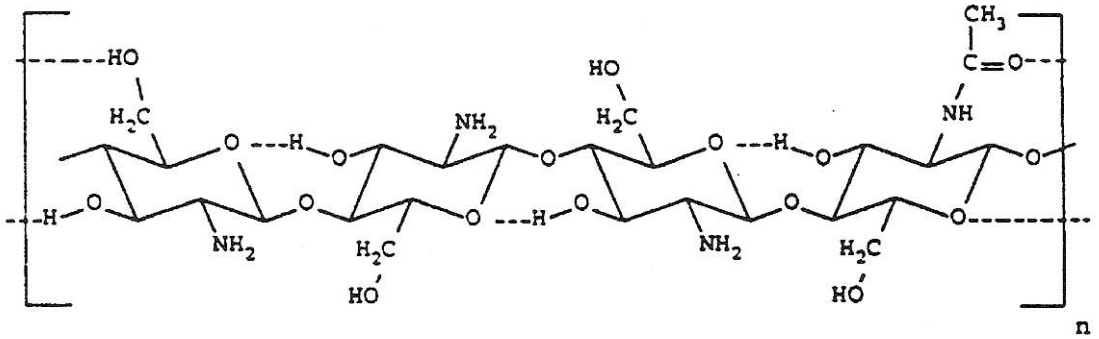
Cellulose



Chitin



Chitosan



Application of Chitosan.

Chitosan has a wide range of uses (Table 2). Low molecular weight chitosans with MW in the range of 5-10 kDa are known to possess strong bactericidal, antitumor activity and also have potential in DNA delivery systems as a DNA carriers (Yumin et al., 2008). The application of chitosan as a food preservative and for other use has been limited by its insolubility at neutral and higher pH. Therefore, in the past two decades, extensive investigations have been carried out to prepare functional derivatives of chitosan and to increase its solubility in water in order to broaden its application (Sugimoto et al., 1998).

A major market of chitin or chitosan is for water purification, where it functions as a flocculent and chelator of trace metal ions. Many of its applications depend on its cationic nature. The amino groups of chitosan are readily available for chemical reactions with acids. These groups can interact with negatively charged colloids. The primary and secondary hydroxyl groups can be used to make commercial derivatives (Li et al., 2008, Chung et al., 2003). Since organic sludge is most commonly negatively charged, a polymer like chitosan is effective for charge neutralisation, agglomeration, and removal of sludge (Muzzarelli, 1977).

Chitosan also appears to be more economically attractive for removal of colors and organic compounds from wastewater than such typical adsorbents as activated carbon (Chung et al., 2003). It has medical applications in the form of bandages, membranes, artificial skin, drug delivery systems, and wound dressings. Everyday products such as contact lenses, cosmetics, photographic paper and fertilizer utilize chitosan (Kumar, 2000). Chitosan has been used as seed coating, and it has been shown to increase crop

yields, (Cuero, 1999). Chitosan triggers a response in the seed that signals the plant to protect itself from natural predators (bacteria and, pathogeic fungi), but it also can stimulate natural microbes that provide protection to certain crops (Cuero, 1999).

It can be also applied in encapsulation technology which is important in the development of cell transplantation techniques for hormone delivery medicine. The semi permeable capsule membrane controls the passage of large molecules and allows small molecules to diffuse through that membrane (Li et al., 1997). According to Li et al. (1997) coagulating agents and flocculants are an important part of chitosan applications because chitosan has a high density of amino groups on the polymer chains that can interact with negatively charged substances, such as proteins and dyes. Furthermore chitosan can be used in the fibre industry.

Park et al. (1996) reported that wool fabrics treated with chitosan showed excellent antimicrobial and deodorant properties. These properties were dependant on the degree of deacetylation and molecular weight. Hirano (1989) categorized the possible applications of chitin derivatives. According to his study, O-acyl derivatives of chitosan can be used as an emulsifier and O-hydroxyl-alkyl derivatives that are water soluble are used as an ingredient in skin care products.

Table 2. Various Applications of Chitin, Chitosan and Derivates

Area of application	**Applications**
Water purification	Removal of heavy metals (Hg, Cd, Pb, Ag, and Ni), pesticides, phenols, dyes
Antimicrobial Agent	Bactericidal, Fungicidal
Chemical industry	Enzyme/cell immobilization, encapsulation of nurtaceuticals, chromatography, analytical reagents
Plant protection	Inhibiton of fungal growth, suppression of plant parasites and pathogens, fertilizer
Food additive	Thickening, emulsifying, and stabilizing agent, color stabbilization
Edible Film	Controlled release of antimicrobials, antioxidants, nutrients, flavours, reverse osmosis membranes, rate of respiration,reduction of oxygen partial pressure,
Nutrition	Dietary fiber, digestive aid, feed supplement for animals
Cosmetic Industry	Cosmetic ingredients for hair and skin cares, contact lenses
Biomedical	Dressing material for the skin burn, bandages, blood antithrombogenic, anticoagulant material
Others	Papermaking additive for surface strength improvement, textile and woven fabrics

Source: Shahidi et al. (1998)

Antimicrobial Activity of Chitosan.

Numerous researchers have studied the antimicrobial activity of chitosan in various fields. It has been confirmed that chitosan has antimicrobial activities against bacteria and fungi. However these inhibitions vary with properties of the used chitosan and the acid or solution in which chitosan is dissolved. For example, the use of liquid chitosan as an antimicrobial agent is more effective than solid form. Liquid chitosan is readily or immediately up taken by microbial and plant cells as compared with slow uptake of the solid (Cuero, 1999). Coma et al. (2002) studied chitosan in a solid form and

found it incapable of diffusing through the adjacent agar media, inhibiting organisms only in direct contact with the active sites of chitosan.

Most studies about the antimicrobial activity of chitosan involve only one or a few different molecular weights of chitosans and chitosan oligomers. No et al. (2001), tested the antibacterial activity of chitosan with different MW. Six chitosans and six chitosan oligomers with different molecular weights (Mw=1671, 1106, 746, 470, 224, and 28 kDa; designated 1–6) and chitosan oligomers (Mw=22, 10, 7, 4, 2, and 1 kDa; designated 7–12) were examined against four gram-negative (*Escherichia coli*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, and *Vibrio parahaemolyticus*) and seven gram-positive bacteria (*Listeria monocytogenes*, *Bacillus megaterium*, *B. cereus*, *Staphylococcus aureus*, *Lactobacillus plantarum*, *L. brevis*, and *L. bulgaricus*).

Chitosans showed higher antibacterial activities than chitosan oligomers and markedly inhibited growth of most bacteria tested. Chitosan with the MW of 746 kDa showed the highest antibacterial activity and completely inhibited the gram positive bacteria, *Listeria monocytogenes, Bacillus cereus* and *Staphylococcus aureus*. Chitosan oligomers of 1 kDa were effective in inhibiting the growth of gram negative bacteria, *Escheria coli, Pseudomonas fluorescens* and *Salmonella typhimurium* and 2 kDa was most effective in inhibiting the gram positive bacteria, *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Staphylococcus aureus, and Lactobacillus brevis*.

The inhibitory effects differed with regard to the molecular weight of chitosan and the type of bacterium. Chitosan generally showed stronger bactericidal effects for gram-positive bacteria than for gram-negative bacteria. Furthermore the antibacterial activity of

chitosan was affected by pH with greater activity being found at lower pH. For example, *E. coli* showed 4.48–5.67 viable cell log numbers at pH 4.5, and 6.40–6.69 at pH 5.9 (No et al., 2001). Yang et al. (2005) reported that the antibacterial activity of chitosan derivatives against *E. coli* increased as the pH increased from 5.0 and reached a maximum around the pH of 7.0–7.5. Wang (1992) confirmed that the inhibitory effect of chitosan varied with concentration and pH. That means a small change in the pH can cause a modified reaction. In a similar study, Sudharshan et al. (1992) reported that the bacterial effect was no longer present at pH 7 due to the presence of a significant proportion of uncharged amino groups and poor solubility of chitosan. According to mentioned studies it could be considered that the minimal inhibitory concentration (MIC) of chitosan and its derivates varies significantly for different bacterial cultures and is influenced by a host of factors such as pH of the growth medium, the degree of polymerization of chitosan and the presence or absence of interfering substances such as lipids and proteins (No et al., 2002).

Further, it can be concluded that comparing MIC values from different chitosan studies is difficult because of possible differences in (1) characteristics (deacetylation and polymerization degree) of the chitosan used in these studies, (2) experimental incubation temperature and pH, (3) chitosan solvent which organic acids being better than inorganic acids and organic solvents with higher carbon numbers having decreased antimicrobial activity (Chung et al., 2003), (4) the MIC definition and (5) strain and species dependency. More studies should therefore be performed with real food products as a matrix (Devlieghere et al., 2004)

Papineau et al. (1991) reported that the biocidal properties of chitosan in relatively 'clean' systems such as distilled water and buffers are a poor indication of likely performance in complex food systems where interactions with other components may modulate the activity of chitosan, as well as of other food preservatives that may be present. Despite these unique antimicrobial properties, the application of chitosan as a food preservative and for other use has been limited by its insolubility at neutral and higher pH (Sugimoto et al., 1998). Therefore in the past two decades extensive research has been preformed to prepare functional derivates of chitosan to increase its solubility in water to broaden its application. However, chitosan should be combined with other active antimicrobial substances to enhance its antimicrobial properties.

For this purpose various acids that occur naturally can be used, such as fruit and vegetable organic acids or lactic, citric, acetic, sorbic or benzoic acids. Also, since chitosan needs to be dissolved in slightly acid solutions, the production of antimicrobial films from chitosan with organic acids is straightforward (Bégin and Calsteren, 1999). Therefore various studies concentrated on the antimicrobial activities of chitosan in several beverages and food such as apple juice (Roller and Covill, 1999), orange juice and milk (Lee et al., 2004), banana fruit (Win et al., 2007), strawberries (Ribeiro et al., 2007), pork sausages (Georgantelis et al., 2006 and Soultos et al., 2008) meat (Darmadji and Zumimoto, 2003, Kanatt et al., 2007) showed promise**.**

The ability of chitosan to inhibit the growth of fungi has effectively been used for practical applications such as seed treatment and fruit and vegetable protection. When chitosan enters the host cells, it triggers a sequence of reactions, thus inducing disease

resistance responses. Hardwiger et al. (1984) studied seed and foliar treatments of field crops with commercial chitosan. They applied seed treatments ranging from 60 – 1000 µg of chitosan per gram of seed on winter and spring wheat, peas, and lentils during a 5-year trial. Plant yield increased 20-30%. Reduction of damp off, a bacterial disease that affects seedling and causes the stems to rot at soil level, and other symptoms of fungal infection were observed.

Therefore it can be concluded that chitosan is a multiple endowed compound with antimicrobial properties affecting growth and physiology of most microorganisms, including algae, fungi, bacteria, protozoa and viruses. The degree of efficiency and the mode of action of chitosan varies according to the microorganisms targeted. Again, the antimicrobial activity of chitosan is influenced by the chemical makeup of the chitosan and the environmental conditions. For practical application of chitosan as an antimicrobial agent it is necessary to establish a fundamental baseline between intrinsic and extrinsic factors with the goal to develop a clear understanding of the biological activity of chitosan (Cuero, 1999).

Antimicrobial Mechanisms of Chitosan.

The exact antimicrobial mechanisms of chitosan are still unclear but there are several proposals on its mode of action. The mechanisms of the antimicrobial activity of chitosan were different between gram-positive and negative bacteria. Additionally, the antimicrobial mechanism of chitosan might differ from that of other polysaccharides because there are positive charges on the surface of chitosan (Zheng and Zhu, 2003). This

is due to the presence of primary amines on the molecule that bind protons according to the equation:

$Chit\text{-}NH_2 + H_3O^+ + \rightarrow Chit\text{-}NH_3^+ + H_2O$

The antimicrobial mechanism of chitosan may depend on the substrate to which it is applied. For example, Ghaouth et al. (1997) and Reddy et al. (2000) reported that chitosan inhibits the growth of several fungi, induces chitinase activity, and elicits phytoalexins and defence barriers in the host tissues. But when applied in systems such as processed food or microbiological media, chitosan apparently directly affects microbial cells (Devlieghere et al., 2004). Sashiwa and Aiba (2004) and Campell (2003) the proposed a mechanism behind this antimicrobial activity and can be summarized as follows:

(1) The cationic nature of chitosan causes it to bind with sialic acid in phospholipids, consequently restraining the movement of microbiological substances.

(2) Oligomeric chitosan penetrates into the cells of microorganisms and prevents the growth of cells by preventing the transformation of RNA from the DNA.

Another major proposal is the reduction of bacterial metabolism by stacking chitosan molecules on the bacterical cell wall (Uchida, 1988). Other research tested two different molecular weight chitosans [MW 9300 and 2200] were tested and it was reported that the accumulation of 9300 MW chitosan was found in the cell wall of *E. coli* (Tokura et al., 1997). The stacking of MW 9300 was confirmed by the use of FTIC (Fluorescein isothioyanate) labelled chitosan oligomer. The permeation of MW 2200 was also observed in the cell wall without stacking on the surface of the cell wall. The

conclusion of their study was that the antimicrobial activity of chitosan seems to be caused mainly by the blocking of the nutrition supply through the cell wall of bacteria. Tokura et al. (1997) also confirmed that the molecular weight of chitosan must be less then or around 5000 so it can permeate into the cell wall. Zheng and Zhu (2003) discovered that for *S. aureus*, a gram-positive bacteria, as the MW of chitosan increased, the antimicrobial effect was enhanced.

The main reason might be that chitosan of higher MW forms a film which inhibits nutrient adsorptions. For *E. coli*, a gram-negative bacteria, as the MW of chitosan decreased, the antimicrobial effect was enhanced. The main reason might be that chitosan of lower MW enters the microbial cell more easily, which disturbs the metabolism of the cell. It has been demonstrated that lower MW chitosans (of less than 10kDa) have greater antimicrobial activity than native chitosans (Uchida et al., 1989).

Factors Affecting the Antimicrobial Properties of Chitosan

The antimicrobial activity is likely to differ based on the preparation methods used to convert chitin into chitosan. The outcome of a test can be affected by factors such as the volume of inoculum, growth phase, culture media used, pH of the media, incubation time and temperature. Comparison of published data is difficult. Also, the antimicrobial action is influenced by intrinsic and extrinsic factors such as the type of chitosan (e.g. plain or derivate), degree of chitosan polymerization, host natural nutrient constituency, substrate chemical, molecular weight, nutrient composition and/or environmental conditions (e.g. substrate water activity (Aw), and/or moisture) (Cuero, 1999).

Furthermore, the antibacterial effect of chitosan and its oligomers is reported to be dependent on its molecular weight or viscosity (Jeon et al., 2001 and No et al., 2002). For example, Cho et al. (1998) reported that the antibacterial activity of chitosan against *Escherichia coli* and *Bacillus* sp. increased with decreased viscosity using an enzymatic hydrolysis from 1000 to 10 cP. No et al. (2002) confirmed that the growth of *E. coli* and *B. cereus* also was inhibited more effectively by chitosan of 746 or 470 kDa than by chitosan of 1671 or 1106 kDa.

Viscosity.

Change in viscosity of chitosan solution during storage may influence its functional properties. No et al. (2006) reported that the viscosity of chitosan solution decreases with increased storage time and temperature. The antimicrobial activity of chitosan solutions (M_w of 2025 and 1110 kDa) against gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and gram-negative (*Salmonella enteritidis* and *Escherichia coli*) bacteria were investigated at 4 and 25 °C after 15-week storage. The viscosity of the chitosan solutions (1% (w/v) in 1% (v/v) acetic and/or lactic acid) decreased with increased storage time and temperature.

After 15-week storage, the decrease in viscosity ranged from 44 to 48% and 81 to 90% of the initial viscosity value, respectively, at 4 and 25 °C. The viscosity of chitosan solution decreased by 44–48% of the initial value at 4 °C and 81–90% at 25 °C after 15 weeks of storage. These results document the instability of chitosan solution under storage conditions of 25 °C. In general, chitosan solutions before storage showed higher antibacterial activity than chitosan solutions after 15-week storage. Thus, it is

recommended that chitosan solutions be freshly prepared if intended for use as an antibacterial agent for improved shelf-life of foods.

pH.

Chitosan with positive charges results in two consequences. First, the intermolecular electric repulsion is increased due to the more positive charges, which leads to a longer persistence length, and prevents chitosan from entering bacterial cells and second, chitosan with positive charges easily reacts with negatively charged bacteria and further inhibits bacterial growth.

The antimicrobial activity of chitosan increases with decreasing pH (Jeon et al., 2001, No et al., 2002, Roller and Covill, 1999, Yang et al., 2005). This is due to the fact that the amino groups of chitosan become ionized at pH below 6 and carry a positive charge which leads to a longer persistence length which prevents chitosan from entering bacterial cells and more interaction with the negatively charged surfaces which inhibits bacterial growth (Chung et al., 2003).

Unmodified chitosan is not antimicrobially active at pH 7, since it does not dissolve and does not contain a positive charge on the amino groups (Chung et al., 2005, and Yang et al., 2005). Sudarshan et al. (1992) reported that chitosan was no longer bactericidal at pH 7 due to the presence of a significant proportion of uncharged amino groups and the poor solubility of chitosan. Jumaa et al. (2002) concluded that a relative small shift in pH can cause a sudden change in the active concentration and can cause a large difference in antimicrobial activity as a consequence.

Deacetylation.

One of the more important chemical characteristics of chitosan is its percent deacetylation (% DA). The deacetylation is the process that allows converting chitin to chitosan by the removal of acetyl groups (Campell, 2003). The abundance of the highly reactive, cationic amine groups determines the % DA of Chitosan. Essentially, the more amine groups present, the more deacetylated the chitosan. Chitosan with a higher % DA requires longer and more strenuous processing. Full deacetylation is nearly impossible to achieve. The term "chitosan" is only applied when the % DA is 70% or higher. Commercial chitosans typically range from 70 to 95% DA. Chitosans with higher % DA usually have lower molecular weights (Vanson, 1995). The degree of % DA can be determined with FTIR, NMR spectroscopy or HPLC (Park, 1998). Functional properties of chitosan, such as thickening, film-formation, metal binding and antimicrobial activity, depend on its molecular weight and degree of acetylation (Muzarelli, 1977, No et al., 2001).

Molecular Weight.

According to Rinaduo and Domard (1987), gel permeation chromatography is an accurate way to determine the molecular weight of chitosan. Molecular weight (MW) relationships to antimicrobial activity by chitosan oligomers have been reported by various investigators. Jeon et al. (2001) reported that MW (10–1 kDa) of chitosan oligomers is critical for microorganism inhibition and the efficacy increased with MW. Unmodified chitosans showed a contradictory molecular weight dependent activity, i.e. the antimicrobial activity against *E. coli* increased with decreasing molecular weight

whereas activity against *S. aureus* increased with increasing molecular weight (Hoppola et al., 2006). The, molecular weight of commercial chitosan is noticeably lower than that of native chitin, which is 1,000,000 Daltons (Da) or higher. Molecular weight of chitosan typically falls between 100,000 and 1,200,000 Da (Campell, 2003). Sekiguchi et al. (1994) determined that 0.2-0.3% chitosan oligomer with MW 11,000 Da suppressed the growth of *B. cereus*.

Preparation of chitosan solutions, especially from high MW chitosans, requires several hours due to its high viscosity. For commercial applications, it would be practical to prepare chitosan solutions in bulk and to store them for further use. However, during storage specific characteristics of chitosan, such as viscosity or molecular weight, may be altered. Thus, change in viscosity of chitosan solution must be monitored since it may influence other functional properties of the chitosan solution (No et al., 2006).

Chitosan Degradation by Irradiation

It has been reported that chitosan can be degraded into lower molecular weights by acidic hydrolysis or enzymatic treatment. Chemical treatment is an easy, low cost process, but chemical waste and reproducibility are the main problems. Enzymatic hydrolysis is an effective way to achieve specific cleavage of chitosan oligomers. However, it requires multisteps, particularly, enzyme preparation and purification of the product. Radiation can provide a useful tool for degradation of different polymers.

In the reaction, no other chemical reagents are introduced and there is not a need to control the temperature, environment or additives (Feng et al., 2008). The most common type of irradiator uses the isotope cobalt^{60} as source of irradiation. To achieve

cobalt^{60}, natural non irradiated cobalt^{59} is tightly compressed into small cylindrical pellets, which are placed in a nuclear reactor where they are constantly bombarded by neutrons for about one year. This process results in pellets of highly purified cobalt^{60} which produce a controlled emission of gamma rays (Satin, 1993).

The cobalt^{60} used in food irradiation is the same as used in medical irradiators and the product of very sophisticated engineering, manufacturing and quality control processes (Satin, 1993). Gamma rays are used in a facility which is specifically designed to irradiate products. The irradiation source Co^{60} is located in the irradiation chamber and is stored in a protective environment. When required, the source is raised out of its shielding so that it can treat the products in question.

The irradiation chamber is constructed with thick concrete walls in order to absorb all gamma rays which are not absorbed by the products. Irradiation sources can also be made with CS ^{137}, an isotope of cesium. Cs^{137} irradiators represent an extremely small proportion of today's irradiators and are not used for commercial irradiation. There are practical reasons why Cobalt^{60} rays are the most preferred. Advantages of Co^{60} irradiators include greater degree of overall efficiency, better gamma ray penetration, and greater environmental safety due to its complete insolubility in water (Satin, 1993).

Feng et al. (2008) studied the antioxidant activity of irradiated chitosan and found that increased dosages (2-20 kGy) showed decreased molecular weight. According to Choi et al. (2002) increasing irradiation dosages caused the color of the solutions to change to a more intense brown and the viscosity decreased rapidly up to 10 kGy and

then slowed down. Nagasawa et al. (2000) concluded that the browning of the chitosan was due to double bond formation by chain scission.

Furthermore a radical mediated lipid peroxidation assay, reducing power, superoxide radicals and hydroxyl radicals assays showed that irradiation of chitosan, especially with the 20 kGy, gives enough degradation to increase the antioxidant activity, with a change of molecular weight. Chitosan irradiated at 20 kGy and with the molecular weight of 2.1×10^3 exhibited high reductive capacity and expressed good inhibition of linoleic acid peroxidation. Matsuhashi and Kume (1999) irradiated chitosan with 100 kGy and showed that the antimicrobial activity of chitosan with a molecular weight of $1 \times 10^5 - 3 \times 10^3$ was most effective in suppressing the growth of *E. Coli*. On the other hand, chitosan whose molecular weight was less than 1×10^5 had no activity.

MATERIALS AND METHODS

Chitosan

Odorless, tasteless, chitosan with 92.06 % deacetylation was obtained from Parchem Trading Ltd., NY in dry powder form. Compared to the chitosan flakes used in previous studies (Campbell, 2003) the chitosan used in this research had a finer particle size (60-80 mesh) and higher deacetylation degree. Glacial acetic acid was purchased from Fisher Chemical (Fair Lawn, NJ).

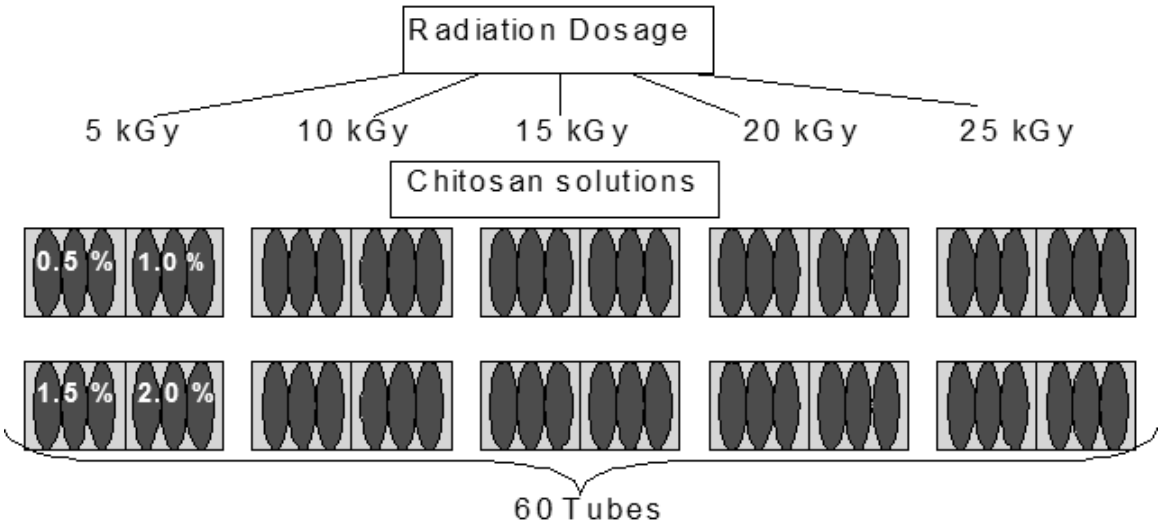
Preparation and Irradiation of Chitosan Solution

A 1 % (v/v) acetic acid concentration was used to prepare all chitosan solutions. A 0.5% chitosan solution was made by adding 297 ml distilled water and 3.0 ml acetic acid to a 1.5 g portion of chitosan. A 1 % chitosan solution was made by adding 297 ml distilled water and 3.0 ml acetic acid to a 3.0 g portion of chitosan. A 1.5 % chitosan solution was made by adding 297 ml distilled water and 3.0 ml acetic acid to a 4.5 g portion of chitosan and a 2 % chitosan solution was made by adding 297 ml distilled water and 3.0 ml acetic acid to a 6.0 g portion of chitosan. Each solution was mixed on a Nuova stir plate (Thermolyne, Dubuque, Iowa) at 90^0C until the chitosan was clearly dissolved.

The chitosan solution was poured through a 90 mm diameter Pyrex® Bucher Funnel with perforated plate and 8 layers of Veratec cheesecloth (BBA Nonwovens, Simpsonville, SC) to filter the solution. After 2 hours cooling at room temperature each solution was filled in Falcon® 50 ml tubes (Becton Dickinson, NJ) and shipped overnight

for irradiation with Co^{60} gamma rays at FTSI INC. (Mulberry, Florida). Irradiation dosages were verified by dosimetry. Figure 5 gives an overview of the different chitosan concentrations and irradiation dosages of the treated solutions.

Figure 5. Irradiation Dosages and Chitosan Concentrations of treated Solutions



Bacterial Culture

The test organism for this study was *Listeria innocua* (ATCC 33090). The culture was stored in brain heart infusion (BHI) broth with 20% glycerol at – 70^0 C . To prepare stock cultures, frozen samples were allow to thaw at room temperature, and 0.1 ml was transferred to 10 ml of BHI broth. After two seconds of mixing by vortex (Reax 2000, Buchler Instruments, Labconco corporation, Kansas City, MO) to ensure resuspension, the stock cultures were grown in a gyrotory water bath shaker (Model G76, New Brunswick Scientific Co., Edison, NJ) at 37^0C for 24 hours.

A second transfer of 0.1 ml of the culture into 10 ml of BHI broth was grown in a water bath shaker at 37^0C for 16 hours. An initial working stock culture was incubated and stored at 4^0C with no agitation for up to 2 weeks and used to re-establish a working stock. To re-establish a culture from a 4^0C stock, the same procedure was used as with the -70^0C stock.

Antimicrobial Activity by Direct Droplet Method

An aliquot (0.3 ml) of the refrigerated bacterial culture was transferred to a tube containing 9 ml of sterile BHI broth. The inoculum was incubated at 37^0C for 16 hours and then centrifuged at 4000 rpm for 25 minutes using a Sorvall Intsruments Centrifuge (DYNAC II Centrifuge, Clay Adams, Becton Dickinson and Company). The pellet was re-suspended in 9.9 ml of sterile 0.1% peptone water. Mixing by vortex (Reax 2000, Buchler Instruments, Labconco corporation, Kansas City, MO) was done to ensure resuspension.

After mixing, the suspension was poured into 99 ml sterile, 0.1% peptone water. The sample was then spiral plated (Autoplate® 4000, Spiral Biotech, Bethesda, MD) onto tryptic soy agar (TSA) plates to achieve even lawns with approximately 10^5 CFU/ml. The MOX agar plates were inoculated with *Listeria innocua* (10^5 CFU/ml) using a sterile spreader. One 0.02 ml droplet of chitosan solution was applied to one half of each plate.

The other half was treated with 0.02 ml of sterile, distilled water. In addition, TSA plates that had not been innoculated with the *Listeria innocua* culture were prepared with 0.02 ml chitosan solution and sterile, distilled water droplets. Plates were kept upright in a laminar flow hood (Delta Series Purifier Class II Biosafety Cabinet, Labonco

Corporation, Kansas City, MO) until the droplets had dried. The plates were incubated at 37^0C for 48 hours and then examined for inhibition of growth. Inhibition of growth was reported as a + for clearing of *Listeria innocua* colonies under the droplet (Table 4). This entire process was completed in triplicate.

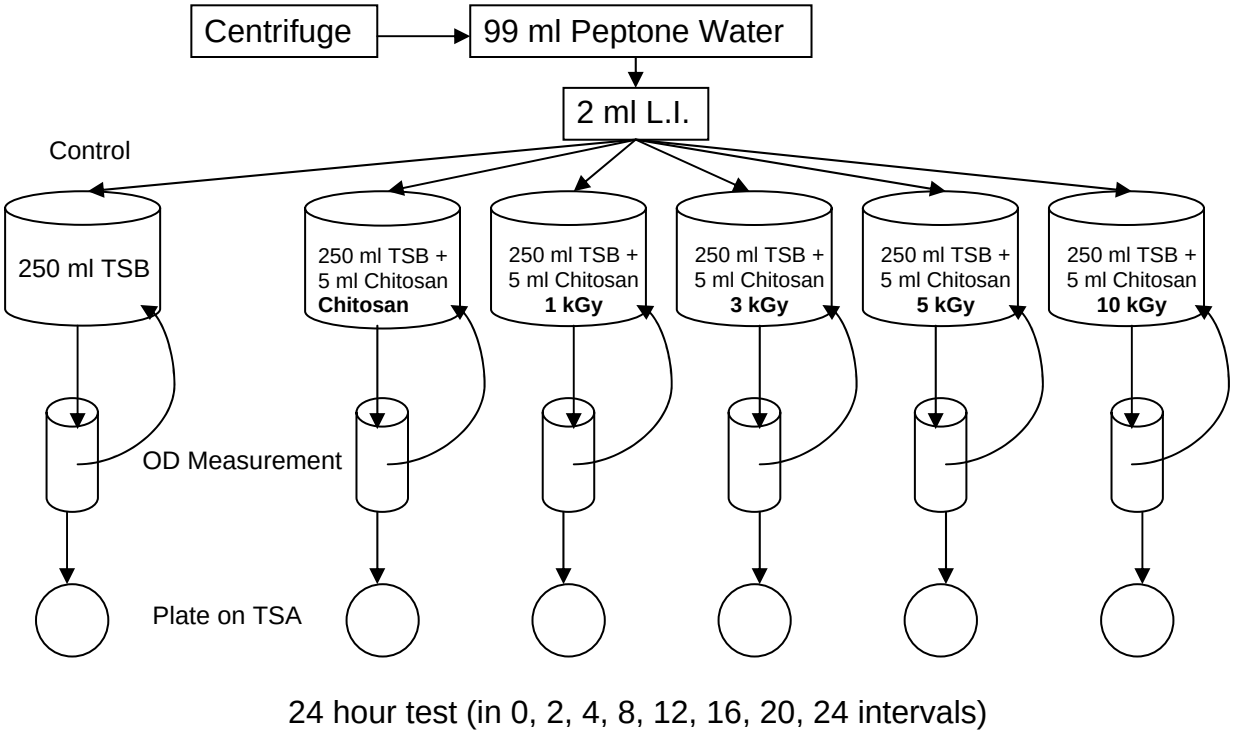
Antimicrobial Activity by Optical Density and Plate Count

Growth curves of *L. innocua* and optical density (OD) measurements were established before running the tests for comparison purposes (Appendix A). The antimicrobial activity of the chitosan solutions was determined by an OD method and by plate counts. An aliquot (0.3 ml) of the refrigerated *L. innocua* inoculum was transferred to a centrifuge tube containing 9 ml of sterile BHI broth. The innoculum was incubated at 37^0C for 16 hours and then centrifuged at 4000 rpm for 25 minutes using a Sorvall Intsruments Centrifuge (DYNAC II Centrifuge, Clay Adams, Becton Dickinson and Company).

The pellet was re-suspended in 9.9 ml of sterile 0.1% peptone water. Mixing by vortex (Reax 2000, Buchler Instruments, Labconco Corp., Kansas City, MO) was done to ensure resuspension. After mixing the suspension into 99 ml sterile 0.1% peptone water, six Erlenmeyer flasks were prepared each with 250 ml tryptic soy broth (TSB) and sterilized 20 minutes at 121^0C. One optical density tube with 6 ml TSB was also sterilized as a blank for the optical density measurements. Five ml from each irradiated 2% chitosan solution (1 kGy, 3 kGy, 5kGy and 10 kGy) and a non-irradiated solution was added to separate flasks.

One flask contained no chitosan as a control. After adding the chitosan, 2 ml of the *L. innocua* suspension was added to each flask and shaken by a gyrotory water bath shaker at 37°C. After shaking, the OD was measured at 600 nm with a Spectrophotometer (Spectronic 20D+, Thermo spectronic). Also, plating samples were taken every 0, 2, 4, 8, 12, 16, 20 and 24 hours. This entire process was completed in quadruplicate. Bacterial counts were determined as colony forming units (CFU) and reported as log CFU/ml. Figure 6 shows a flowchart of the OD and plating methods.

Figure 6. Optical Density and TSA Plating Methods for Measuring Antimicrobial Activity of Irradiated Chitosan Solutions on *L. innocua* (24 hours test).



Statistical analysis.

Each experiment was performed in quadruplicate. The data were analyzed by ANOVA using SAS (Version 9.1, SAS Institute Corp., Cory, NC) and differences among mean values were determined by Duncan's multiple range test. Significance was defined at ($p < 0.05$). Microsoft Windows Excel was used for plotting diagrams from statistical data.

Chitosan Coating Method

Irradiated and non-irradiated chitosan solutions were coated on a Cryovac® HangPak™ B2000 (coextruded multilayer polyolefin/nylon structure). A flow chart summarizing the coating preparation procedure is shown in Figure 7. The surface tension of the inside layer of the film was determined with ACCU DYNE TEST™ pens to be 56 dyne/cm (+/- 2.0 dyne/cm). The inside layer of the film was used for further experiments because of a higher surface tension than the outside layer.

The coated films were cut to a diameter of 260 x 200 mm to fit the coater and taped on cardboard for an even coating. To increase the surface tension of the film, a laboratory hand corona treater (Model BD-20C, Electro – Technic Products Inc., Chicago, IL) was used. The Cryovac® multilayer film was coated with a hand coater (CSD Laboratory drawdown machine, Model II, Consler Scientific, Oldsmar, FL) by dropping the solution close in front of the Mayer rod 20 and moving it constantly from the top edge to the bottom edge of the film (Figure 8).

An even coating of 2 % chitosan solution was spread onto the Cryovac® multilayer films. The films were dried approximately 24 hours at ambient conditions

under a laminar flow hood. The chitosan coated films were identified by the solution from which each film was coated. For example, a film prepared by coating a 1 kGy solution was referred to as a 1 kGy film.

Figure 7. Flowchart of Chitosan Film Coating

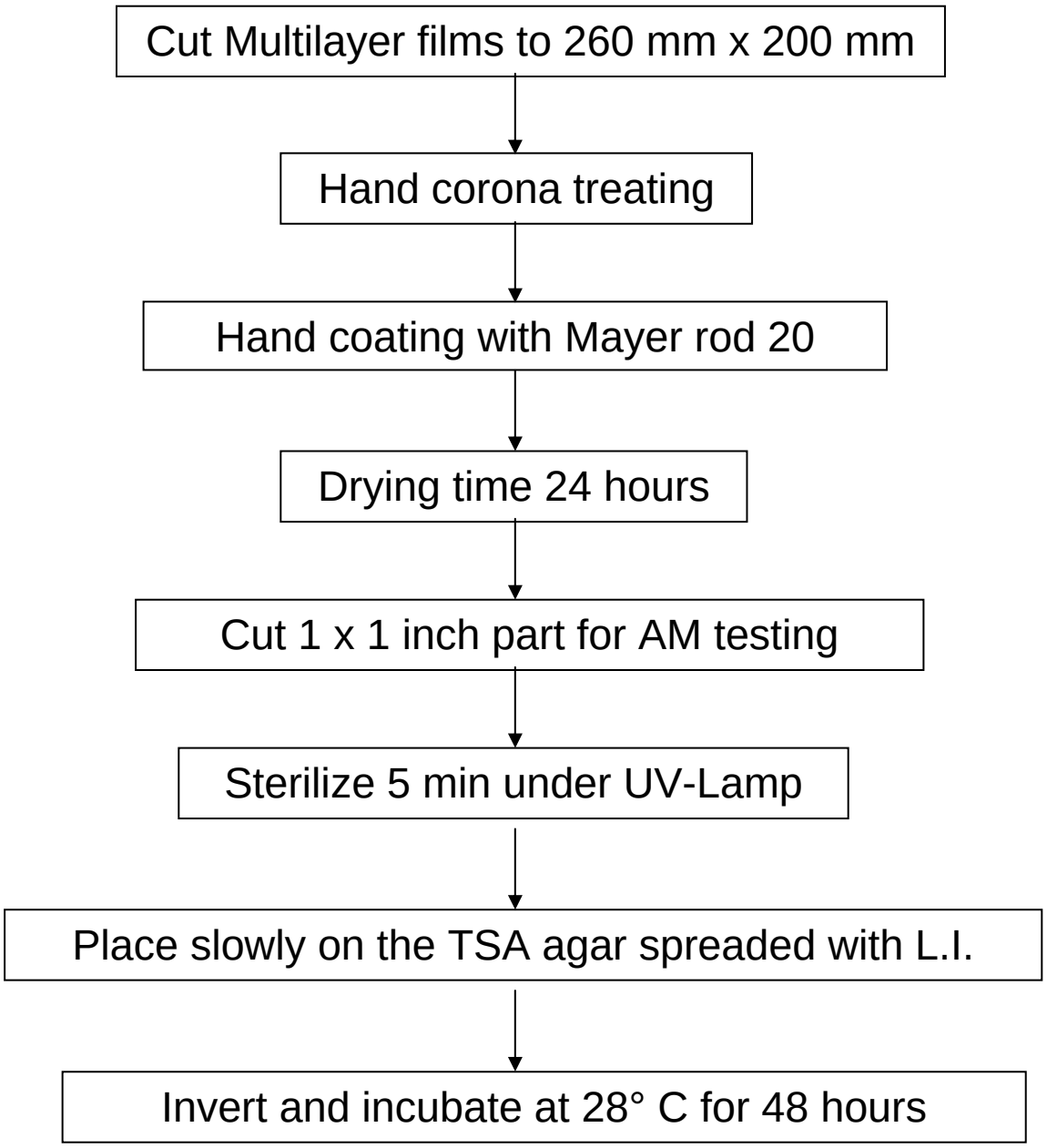


Figure 8. Hand Coater with Mayer Rod and Corona Treater

To determine the evenness of coating chitosan on the multilayer film, 6 ml of 0.01 % iodine solution was placed in front of a small sized Mayer Rod 5 and spread over the coated films. After 12 hours drying, coated and non-coated areas of the film were visible. To measure the exact amount of the chitosan coatings, a 1x1 inch section of the chitosan coated film was weighed (without iodine) with a scale (Denver Instrument, APX-20), the chitosan was washed off the film with 6 % (v/v) acetic acid, dried for one hour and then reweighed. To compare the antimicrobial properties of increased chitosan amounts on the coated film, the same washing procedure was performed with the same multilayer film coated 4 times with irradiated and non-irradiated chitosan solutions.

Film on Lawn Method

Samples measuring 1 x 1 inch were cut from the coated films and sterilized with a UV light (Zeta 7400, Loclite Corp.) for 5 min. This step helped to eliminate the possibility of bacterial contamination on TSA plates and assured that only *Listeria innocua* colonies would be present. The plates were inoculated with *Listeria innocua* (10^5 CFU/ml) using a sterile spreader. Triplicate sets of the coated film samples were placed on the plates with the coated side down so that the chitosan surface could be in direct contact with the TSA agar. The plates were incubated at 37°C for 48 hours and afterwards examined for inhibition of growth (Figure 7). The zones of inhibition were measured both horizontally and vertically using a micrometer. The average of the two measurements was then recorded for each rectangular area of *L. innocua* inhibition.

Paper Disks on Lawn Method

The disk method was originally devised to test microbial susceptibility to antibiotics. In this method a disk carrying a specific drug concentration is placed on the surface of a solidified medium inoculated with viable cells (Cooper, 1963). In this experiment, circular paper disks (5.5 cm Filter Paper, Baxter, IL) were cut into a diameter of 25 mm. This entire experiment was performed under a laminar flow hood (Delta Series Purifier Class II Biosafety Cabinet, Labonco Corporation, Kansas City, MO) to avoid contamination. The paper disks were weighed and then dipped into non-irradiated and irradiated chitosan solutions (1, 3, 5, 10 kGy).

To determine the amount of absorbed chitosan, the disks were placed in sterile petri dishes and dried for 24 hours in a vacuum oven (Isotemp, Model 285 A, Fisher

Scientific) inducing the vacuum pump (Gast Model 0523, Manufacturing Inc., Michigan, US) after 16 hours, and then weighed again. The same microbial testing methods described for the previous film on lawn method were used. The initial bacterial population was 10^5 CFU/ml. The paper disks were placed onto the surface of the *L. innocua* inoculated TSA agar. The plates were inverted, incubated at 37°C for 48 hours and afterwards examined for inhibition of growth. The zones of inhibition were measured using a micrometer.

Experiments were also performed with wet circular 25 mm paper disks dipped in chitosan solutions and placed in direct contact with *L. innocua* spreaded TSA agar plates. To determine the absorbed amount of chitosan by the paper discs, circular, 5.5 cm paper disks were weighted before and after dipping and drying 24 hours in a vacuum oven. The dipping procedure was repeated twice more to increase the amount of absorbed chitosan.

Determination of Weight Average Molecular Weight of Chitosan

The average weight molecular weight (MW) was determined for the unirradiated chitosan control solution and chitosan solutions irradiated at 1, 3, 5, 10, 15 and 20 kGy. All chitosan solutions contained 2% acetic acid and were filtered through cellulose acetate membranes (3.0 μm pore size, Whatman International Ltd.). The samples were analyzed by high performance size exclusion chromatography coupled to multiangle laser light scattering and refractive index detection (HPSEC-MALLS-RI) system.

The HPSEC-MALLS-RI system consisted of a pump (model 321, Gilson, Middleton, WI, USA) an injector valve with a 200 μl sample loop (model 7725i, Rheodyne, Rohnert Park, CA, USA), a guard column (HyperGel, Thermo Fisher

Scientific Inc., Waltham, MA, USA), a SEC column (HyperGel AP50 7.8×300mm, Thermo Fisher Scientific Inc.) a multiangle laser light scattering detector (HELEOS, Wyatt Technology Corp., Santa Barbara, CA, USA), and a refractive Index detector (RI-150, Thermo Electron Corp., Yokohama City, Japan). The aqueous solution of 0.2 M acetic acid and 0.1 M sodium acetate was used as a mobile phase at a flow rate of 0.4 mL/min.

The normalization of the MALLS detector and the determination of volume delay between MALLS and RI detectors were carried out with bovine serum albumin (BSA). Measurements were carried out at 25°C where the intensity of the scattered light from a polymer solution can be calculated by a Zimm's plot (Barth and Mays, 1991):

$$\frac{Kc}{\Delta R_\theta} = \frac{1}{M_w}\left[1 + \frac{16\pi^2\left\langle r_g{}^2\right\rangle}{3\lambda^2}\sin^2\left(\frac{\theta}{2}\right)\right] + 2cA_2 + \ldots \qquad (1)$$

where ΔR_θ is the excess Rayleight ratio of solution at scattering angle θ, λ the wavelength of the incident light in a vacuum, A_2 the second virial coefficient which describes the polymer interaction in (mol/g²), $\left\langle r_g{}^2\right\rangle$ the mean square radius of gyration of the polymer (molecular radius), c the concentration of the polymer, K the polymer optical constant calculated by refractive index

$$K = \frac{4\pi^2 n_0^2 (dn/dc)^2}{\lambda^4 N_A} \qquad (2)$$

where n_0 is the refractive index of the solvent, N_A is the Avogadro's constant and the dn/dc value the differential index of refraction. The dn/dc value was set to 0.162 for chitosan polymers (Pa and Yu, 2001).

Zimm plots can be constructed with data of the LS signals by plotting the ratio Kc/ΔR_θ against $\sin^2\left(\frac{\theta}{2}\right)$ where the curve is extrapolated to zero concentration and zero scattering angle in Equation (1). The MW and Rg of chitosan polymers were calculated from the data collected from MALLS and RI detectors using ASTRA 5.3 software.

RESULTS AND DISCUSSION

Molecular Weight of Irradiated Chitosan

Molecular Weights.

To establish the effect of irradiation on the MW of chitosan, MW was determined using HPSEC-MALLS-RI. Molecular weight reduction of chitosan occurred with increasing irradiation dosage (Table 3). The irradiation of chitosan also caused a color change to a more intense brown with increasing dosages of irradiation. These results were similar to the studies of Feng et al. (2007) and Choi et al. (2002) who also noticed a colour change of the irradiated sample solutions. The MW of native chitosan has been previously determined to be approximately 210 kDa (Feng et al., 2007).

Table 3. Weight-Average Molecular Weight (Mw) of Chitosan Samples Measured with HPLC-MALLS-RI

Sample Solution	Irradiation Dose (kGy)	Mw (kDa)
Control	0	114.8 ± 2.8
SS-1	1	47.2 ± 4.5
SS-3	3	32.9 ± 0.8
SS-5	5	30.4 ± 1.3
SS-10	10	22.4 ± 0.5
SS-15	15	20.1 ± 0.7
SS-20	20	17.4 ± 0.3

Results are expressed as the mean ± SD (n=4)

Inhibition Testing by Direct Droplet Method.

The inhibition of *Listeria innocua* by 24 different types of chitosans varying in irradiation dosages and chitosan percentages was determined. The irradiation dosages were 5, 10, 15, 20 and 25 kGy and the percentages of chitosan were 0.5, 1.0, 1.5 and 2 % (Table 4). Inhibition occurred only at the drop/medium interface within a 18-20 mm diameter of the applied chitosan droplet. Moreover, no additional inhibitory effect was observed outside the area of the droplet, indicating that inhibition only occurred upon direct surface contact with the liquid chitosan solution.

Chitosan did not appear to diffuse from the droplet area into the surrounding media (TSA or MOX agar). In addition, there was no significant evidence that irradiated chitosan showed greater inhibition than non-irradiated. However, it was found that lower percentages of chitosan (0.5%) in combination with higher irradiation dosages showed decreased inhibition of *L. innocua* on TSA agar. The 0.5% chitosan solution irradiated over 10 kGy showed no inhibition compared to lower irradiation dosages.

It might be suggested that chitosan loses its antimicrobial properties with increasing irradiation dosage and thus decreasing MW, depending on the chitosan concentration. Those irradiation dosages that did not show any inhibition (greater than 10 kGy) were eliminated from further studies. Further experiments only included chitosan solutions irradiated at 1, 3, 5 and 10 kGy. The growth of *L. innocua* was observed elsewhere on the plates, including under the control droplet of sterile distilled water and sterile distilled water with 1% acetic acid. This confirmed that inhibition was solely due

to the presence of chitosan and not masked by the presence of acetic acid, by the presence of another substance, or by lack of oxygen.

Table 4. Inhibition of *Listeria innocua* on TSA Agar by Irradiated Chitosan Solutions [1, 2]

Irradiation Dosage in kGy							**Controls**		
Chitosan Concentration in %		5	10	15	20	25	Non irradiated chitosan	1 % Acetic acid	Dest. Sterile Water
	0.5	+	-	-	-	-	+	-	-
	1.0	+	+	-	-	-	+	-	-
	1.5	+	+	-	-	-	+	-	-
	2.0	+	+	-	-	-	+	-	-

[1] Inhibition is shown by + and no inhibiton is shown by –
[2] All treated samples were in 1 % acetic acid

Comparison of TSA and MOX agars with the experimental group of chitosan solutions showed the ability of MOX agar to improve both visualization and measurement of the resulting inhibition. The color change MOX undergoes when exposed to *Listeria innocua* was very beneficial when trying to measure the zones of inhibition. The lithium salt contained in MOX agar makes it selective for *Listeria*. This inhibitory effect MOX agar has toward other bacteria also better revealed the inhibition of *Listeria innocua* when compared to TSA agar.

The MOX agar plates also showed that chitosan solutions irradiated with irradiation dosages over 10 kGy (less than 22.4 kDa) completely lost their antimicrobial activity against *L. innocua* (Table 5). Irradiation dosages that did not show any inhibition were eliminated from further studies. Irradiation at 10 kGy dosage was determined as a maximum level for further experiments. It was concluded that chitosan with a MW below

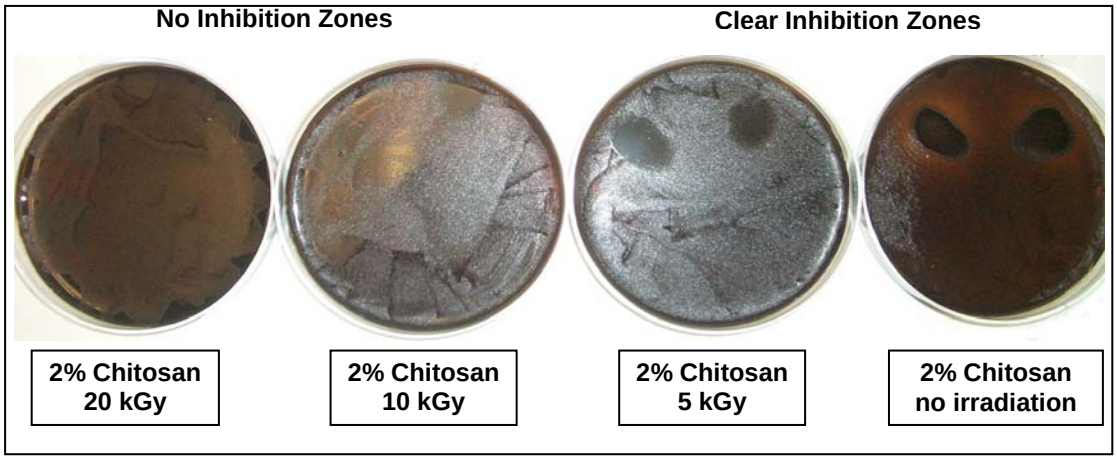
22.4 kDa would have minimal or no inhibitory effect on *L. innocua*. Figure 9 compares 2% chitosan solutions with different irradiation dosages and shows that antimicrobial activity of chitosan decreases with irradiation dosage. The antimicrobial activities of chitosan are believed to originate from its polycationic nature (Cuq et al., 1995, Cuero, 1999). It may be possible that chitosan lost its polycationic properties with higher irradiation dosages because of lower molecular weight.

Table 5. Inhibition of *Listeria innocua* on MOX Agar by Irradiated Chitosan Solutions [1, 2]

Irradiation Dosage in kGy							**Controls**		
Chitosan Concentration in %		5	10	15	20	25	Non irradiated chitosan	1 % Acetic acid	Dest. sterile Water
	0.5	-	-	-	-	-	-	-	-
	1.0	-	-	-	-	-	+	-	-
	1.5	+	-	-	-	-	+	-	-
	2.0	+	-	-	-	-	+	-	-

[1] Inhibition is shown by + and no inhibition is shown by –
[2] All treated samples were in 1 % acetic acid

Figure 9. Decreasing Inhibition Zones on MOX Agar with increasing Irradiation Dosage



Antimicrobial Activity by Optical Density and Plate Count

Optical Density.

The OD was measured 24 hours before plating the samples on TSA agar. Results in Figure 10 showed that all OD curves for the chitosan solutions, especially non treated chitosan, were below the control OD curve after 8 hours. Comparing these results to the average bacterial count (Figure 11) there is significant evidence to conclude that all chitosan solutions inhibited *L.innocua*. The OD curves showed that chitosan samples initially had a higher OD count attributed to turbidity in the test tubes and the slow growing rate of the bacteria in the start phase. After 16 hours a clear separation of the chitosan curves was noticeable. As shown in Figure 10, chitosan irradiated at 10 and 5 kGy and non-irradiated chitosan showed the strongest inhibition.

According to Fernandez-Saiz et al. (2008), previous published works on the antimicrobial capacity of chitosan films showed alterations or even a lack of inhibition when evaluated by optical density (Devlieghere et al., 2004 and Liu et al., 2006). In this

study turbidity was detected in the test tubes containing the nutrient broth (TSB) and chitosan as in the study of Fernandez-Saiz et al. (2008). When 5 ml of the chitosan solution was added to the flask with 250 ml TSB broth, the chitosan precipitated out by flocculation. According to Fernandez-Saiz et al. (2008) this is due to the migration of protonated glucosamine fractions of chitosan into the culture solution. This additional turbidity causes an overestimation of bacterial final concentrations when calculated by optical density.

Therefore, for the present work, a bacterial count was also performed using the TSA agar plate method to better determine the exact inhibition by different chitosans.

Figure 10 Optical Density Measurements for Irradiated and Non-irradiated Chitosan Solutions over a 24 Hours Timeframe.

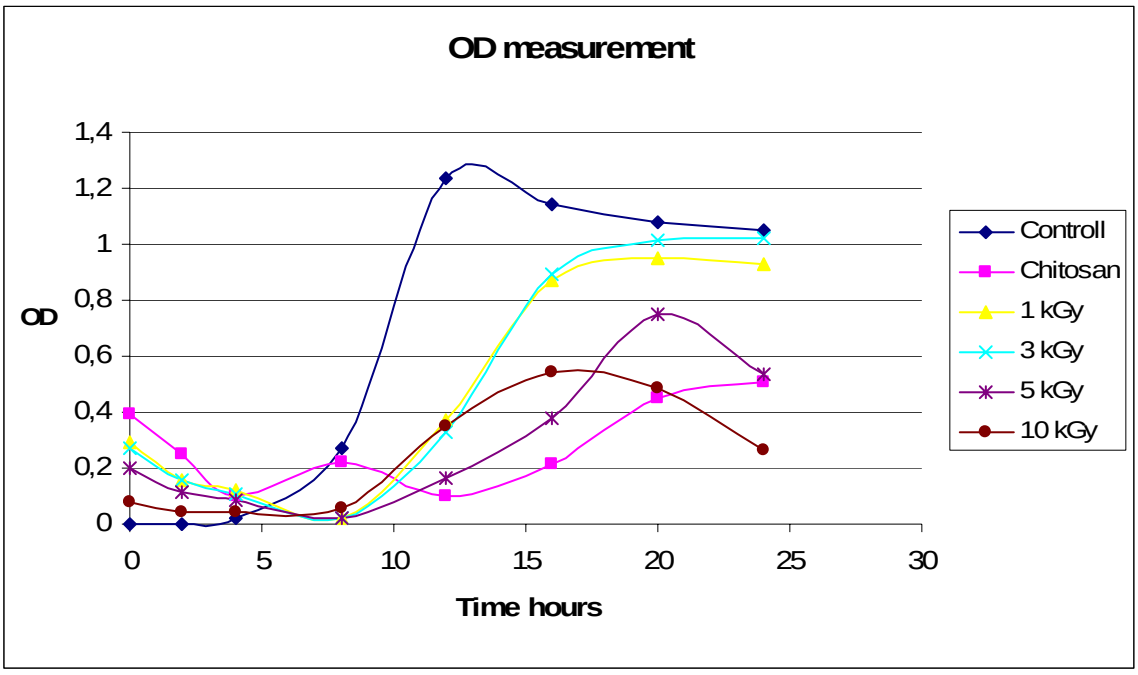
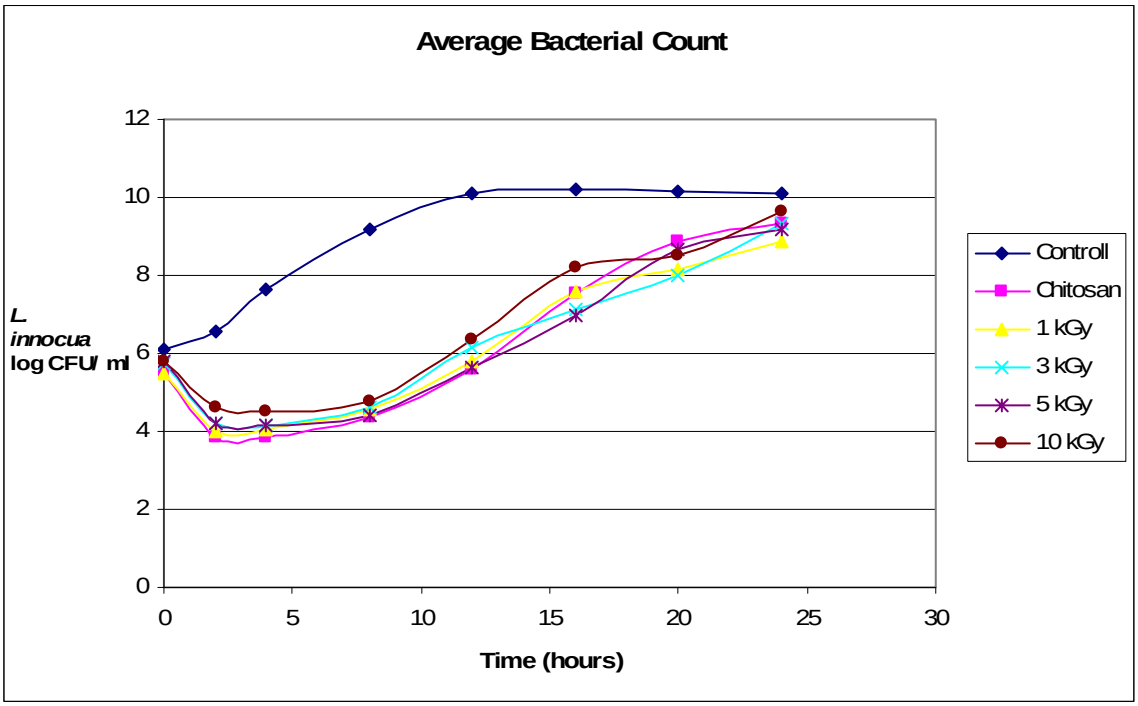


Plate Counts.

The TSA agar plate counts from the shake flasks were determined over a 24-hour time period (Figure 11). There seemed to be no significant difference among the irradiated and non-irradiated chitosans (the difference was less than 1 log). All tested chitosans inhibited *L. innocua* directly after inoculation after 2 hours by 2 log (CFU/ml) and showed strong antimicrobial inhibition between 4 and 12 hours (approximately 4 log). The maximum reduction occurred after 8 hours (4.5 log). However, Figure 11 shows that chitosan as well as LMW chitosan did not kill *L. innocua* completely, but only reduced the bacterial count so that after 12 hours *L. innocua* recovered and regrew. The mean total plate counts for irradiated as well as non-irradiated chitosan (Tabele 5) increased continuously after 12 hours, and remained slightly below the control curve after 24 hours (Figure 11).

Figure 11 Inhibition of *L. innocua* (log CFU/ml) by Irradiated and Non-irradiated Chitosan Solutions over a 24 hour Timeframe



Statistical differences among mean values were analyzed by the Duncan multiple range test (Appendix B). Table 6 displays the results of the Duncan multiple range test combination with mean values and standard deviations over a 24 hour timeframe. There were no significant differences among the chitosan solutions. Furthermore, the test pointed out that the only significant differences were between the chitosan solutions and the control solution (without chitosan).

Table 6. Mean Total Plate Counts of *L. innocua* Inhibition (log CFU/ml) for Different Irradiated and Non-irradiated Chitosan Solutions (24 hours test), Including Standard Deviations and Comparisons among Different Chitosan Solutions with Duncan's Statistical Analysis

	Time in hours							
	0	**2**	**4**	**8**	**12**	**16**	**20**	**24**
Control	6.09± 0,39[1a]	6.54± 0,47 [a]	7.65± 0,40 [a]	9.20± 0,52 [a]	10.11± 0,07 [a]	10.20± 0,02 [a]	10.16± 0,01 [a]	10.09± 0,01 [a]
Chitosan	5.48± 0,30 [b]	3.84± 0,24 [b]	3.86± 0,39 [b]	4.37± 0,68 [b]	5.60± 1,18 [b]	7.52± 0,78 [b]	8.88± 0,19 [b]	9.36± 0,36 [b]
1 kGy	5.50± 0,51 [b]	3.99± 0,52 [b]	4.07± 0,23 [b]	4.56± 0,82 [b]	5.80± 0,66 [b]	7.57± 0,72 [b]	8.16± 0,62 [b]	8.89± 0,23 [b]
3 kGy	5.72± 0,61 [b]	4.18± 0,48 [b]	4.14± 0,31 [b]	4.63± 1,08 [b]	6.16± 1,02 [b]	7.14± 1,65 [b]	7.99± 0,89 [b]	9.34± 0,22 [b]
5 kGy	5.82± 0,26 [b]	4.18± 0,58 [b]	4.14± 0,55 [b]	4.40± 0,56 [b]	5.63± 0,54 [b]	6.96± 1,11 [b]	8.65± 1,01 [b]	9.16± 0,45 [b]
10 kGy	5.77± 0,56 [b]	4.63± 0,46 [b]	4.49± 0,66 [b]	4.77± 1,09 [b]	6.35± 1,03 [b]	8.19± 1,10 [b]	8.50± 0,97 [b]	9.65± 0,45 [b]

[1] Results are expressed as the mean ± SD (n=4)

a-b: The different letters within the same row differ significantly ($p < 0.05$)

It is interesting to note that the two main radiation induced reactions that could significantly affect the usefulness of a polymer are main chain scissions and the formation of crosslinks. In the case of chitosan irradiated with up to 25 kGy, cross-linking has been reported to be negligible but scissions of the 1-4 glycosidic bonds caused a reduction in molecular weight of the polymer (Lim et al., 1997). It has been reported that irradiation improves the antioxidative activity of chitosan (Feng et al., 2008). Their research showed that the antimicrobial activity of chitosan was not affected by irradiation except possibly at very high dosages.

Furthermore, the antimicrobial activity of chitosan depends on many intrinsic and extrinsic factors, for example, degree of deacetylation, molecular weight and source of chitosan. However, Rhoades & Roller (2000) reported that highly degraded products of chitosan by various methods exhibited very low or no antimicrobial activity in laboratory media. This related research also speculated that an antimicrobial compound with powerful antioxidant property has a lower antimicrobial property because the antioxidant property can protect the bacteria.

Antimicrobial Activity of Chitosan Coated Films and Paper

Chitosan Coating and Film on Lawn Assay.

The surface tension of the Cryovac® multilayer film was reduced with a hand corona treater and showed adhesion of the different chitosan solutions used in this study. Coating results with different Mayer rods (10, 20, 30 and 40) showed that the Mayer rod 20 provided strong and even adhesion on the multilayer film. In addition, it was difficult to release the chitosan with water from the film matrix but the chitosan coated film was

washable with acetic acid for exact weight determination purposes. The averages of the measurements of each chitosan film were calculated and are shown in Table 7. To determine the exact amount of chitosan on the each coated film, the average weight of each sample before washing was subtracted from the average weight after washing. The chitosan coating was determined to be 0.71 mg/in².

Table 7. Average Weight Measurements of Irradiated and Non-irradiated Chitosan Coated Solutions with a Mayer 20 Rod (± Standard Derivation)

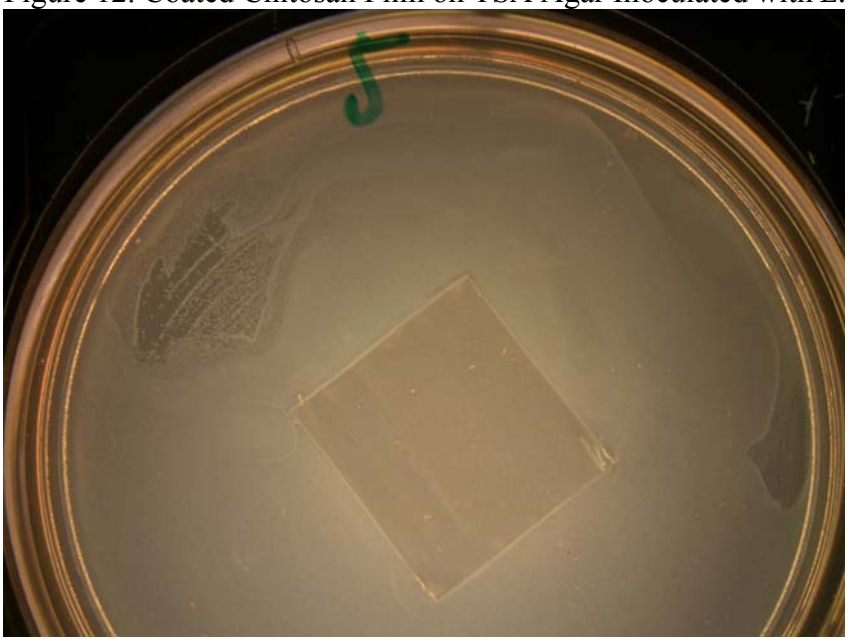
Coated film weight (mg/in²) before washing with 6 % (w/v) acetic acid					
Control	Chitosan	1 kGy	3 kGy	5 kGy	10 kGy
33.1 ± 1.5[1]	34.2 ± 2.1[1]	38.7 ± 2.0[1]	33.3 ± 3.0[1]	37.0 ± 2.4[1]	34.1 ± 3.3[1]
Coated film weight (mg/in²) after washing with 6 % (w/v) acetic acid					
32.9 ± 1.5[1]	33.5 ± 2.0[1]	37.9 ± 1.9[1]	32.4 ± 3.0[1]	36.1 ± 2.4[1]	33.8 ± 3.2[1]

[1] Results are expressed as the mean ± SD (n=3)

Accordingly, it was possible to cut a 1x1 inch sample of the chitosan coated film for the film on lawn assay to determine if zones of inhibition resulted from chitosan diffusion from the films. Triplicate results of the film on lawn assay showed no inhibition zones around the film. Also, it appeared that growth occurred under the films which would indicate that the bacteria were not affected by direct contact to the chitosan film. This result includes all irradiated and non – irradiated chitosan solutions, such as LMW chitosan film irradiated at 5 or 10 kGy. Migration of LMW chitosans outside the film could not be determined. Figure 12 shows a 5 kGy chitosan coated film placed on TSA

agar inoculated with *L. innocua*. This picture is representative for all chitosan coated films used in this experiment. It was considered that the average coating of 0.71 mg/in² may not be sufficient to inhibit bacterial growth, so films were coated with higher levels of chitosan for testing.

Figure 12. Coated Chitosan Film on TSA Agar Inoculated with *L. innocua*



Irradiated and non-irradiated chitosan solutions were coated 4 times on Cryovac® multilayer film resulting in a coating amount of approximately 2.62 mg/in² (Table 8). Again, all chitosan films showed no zones of inhibition after placing on TSA agar inoculated with *L. innocua* and incubating 48 hours at 37°C. It can be concluded that increasing the amount of chitosan coating on the Cryovac® multilayer film showed no diffusion and no zones of inhibition against *L. innocua*. It was also found that the chitosan coating was proportional to the number of coatings.

Table 8. Average Weight Measurements of Irradiated and Non-irradiated Chitosan Coatings with a Mayer 20 Rod after 4 times Coating and drying (± Standard Derivation)

Coated film weight (mg/in²) before washing with 6 % (w/v) acetic acid					
Control	Chitosan	1 kGy	3 kGy	5 kGy	10 kGy
29.5 ± 0.25[1]	29.2 ± 3.3[1]	37.2 ± 1.0[1]	34.2 ± 3.1[1]	32.8 ± 2.6[1]	27.3 ± 5.3[1]
Coated film weight (mg/in²) after washing with 6 % (w/v) acetic acid					
29.4 ± 0.30[1]	26.5 ± 3.1[1]	34.5 ± 1.2[1]	31.8 ± 3.8[1]	30.3 ± 3.2[1]	24.5 ± 5.1[1]

[1] Results are expressed as the mean ± SD (n=4)

Related research results concerning inhibition zone assays on solid medium against *Listeria monocytogenes* showed no clear inhibition zones, regardless of the chitosan content. The poor inhibitory activity of the chitosan coating could be explained by the limitation of the diffusion of chitosan in agar medium (Coma et al., 2002).

Chitosan Coating and Paper Disk on Lawn Assay.

Paper discs were dipped in 1, 3, 5 and 10 kGy irradiated and non–irradiated chitosan solutions to supplement the previous film on lawn study. After 24 hours drying, the discs were placed on *L. innocua* spreaded TSA plates and incubated for 28 hours at 37°C. Results of this test were the same as the previous study with coated films. No zones of inhibition were observed from any of the chitosan samples. Figure 13 shows no diffusion of chitosan into the TSA agar and no inhibition of *Listeria innocua* around the disc. This picture is representative for all dried paper discs used in this experiment. Dipping the paper discs twice in the chitosan solution and drying showed an increase in

absorbed chitosan (Table 9). Likewise, no zones of inhibition were observed with the paper discs dipped twice in chitosan solutions. The average amount of absorbed chitosan in the paper discs was found to be 24 mg / disc.

Table 9. Average Weight Measurement of Chitosan Paper Discs (25mm Diameter) before and after dipping in Chitosan, and drying.

Paper discs without chitosan in g						
Blank	Acid	Chitosan	1 kGy	3 kGy	5 kGy	10 kGy
0.169 ± 0.002[1]	0.166 ± 0.003[1]	0.169 ± 0.003[1]	0.169 ± 0.004[1]	0.172 ± 0.002[1]	0.175 ± 0.004[1]	0.173 ± 0.001[1]
Paper discs dipped 1st time in chitosan and dried 24 hours in vacuum oven in g						
0.169 ± 0.002[1]	0.174 ± 0.004[1]	0.182 ± 0.003[1]	0.182 ± 0.002[1]	0.184 ± 0.001[1]	0.188 ± 0.004[1]	0.184 ± 0.001[1]
Paper discs dipped 2nd time in chitosan and dried 24 hours in vacuum oven in g						
0.169 ± 0.002[1]	0.200 ± 0.003[1]	0.197 ± 0.003[1]	0.194 ± 0.002[1]	0.194 ± 0.002[1]	0.165 ± 0.005[1]	0.169 ± 0.002[1]

[1] Results are expressed as the mean ± SD (n=3)

The results from the coated chitosan films and the dry paper discs were in contrast to wet paper discs. Placing wet chitosan soaked paper discs with the same procedure on TSA agar showed small inhibition zones (Figure 14).

Figure 13. Dry Paper Disk with a Diameter of 25 mm dipped in 10 kGy Chitosan Solution after drying

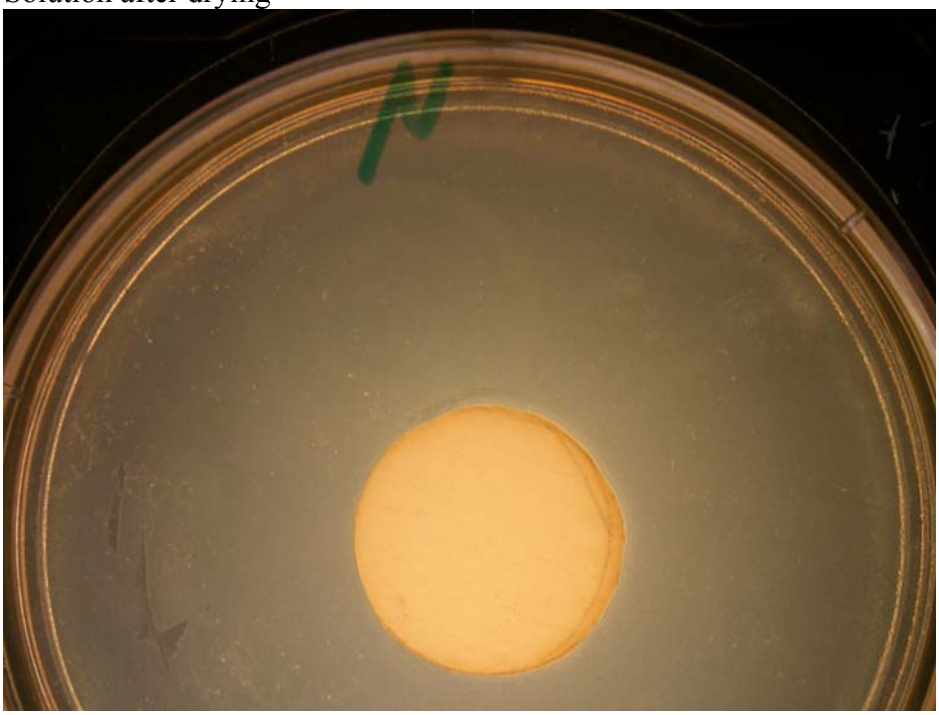
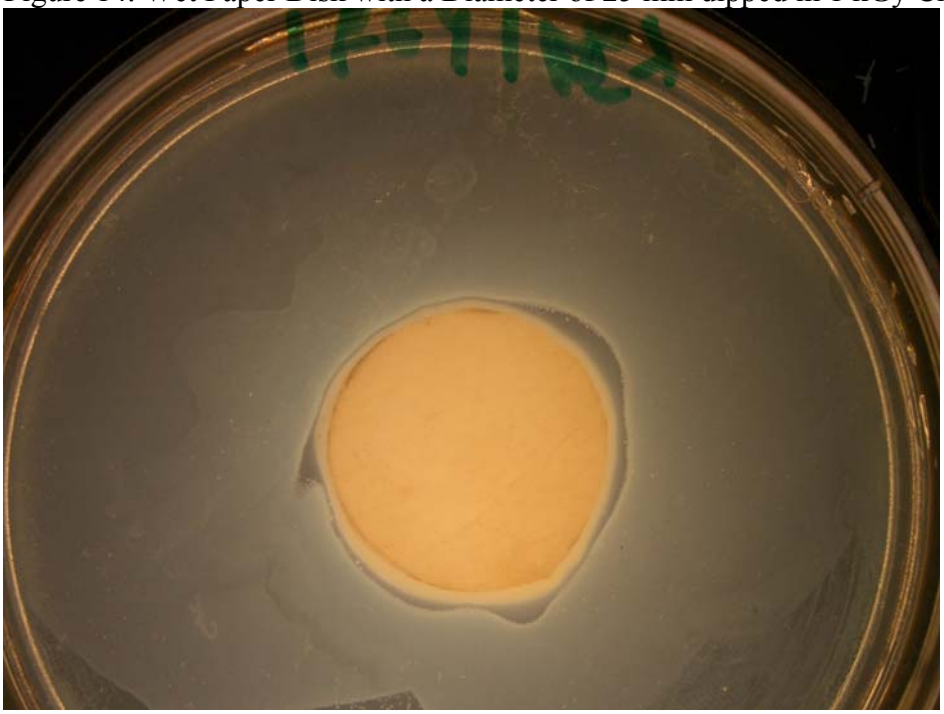


Figure 14. Wet Paper Disk with a Diameter of 25 mm dipped in 1 kGy Chitosan Solution



Thus, no diffusion into the surrounding media could be distinguished for any of the chitosan coated films or paper infused with chitosan. Comparing the results of film on lawn tests as well as wet/dry paper disc tests indicated that chitosan has antimicrobial activity which is effectively expressed only in aqueous systems regardless of the molecular weight of chitosan. Wet paper discs showed diffusion of chitosan into the surrounding agar.

These observations are in accordance with previous work that showed similar growth of *L. monocytogenes* in salmon samples wrapped in chitosan coated film. It was found that antimicrobial properties of chitosan may become negligible when chitosan is in the form of insoluble films. It is possible that chitosan is ineffective in films because it is unable to diffuse through a rigid food matrix such as salmon (Ye et al., 2008).

Other published work using the agar diffusion method showed similar results. Zivanovic et al. (2005) evaluated the antimicrobial capacity of chitosan films enriched with essential oils by the agar diffusion test. These authors concluded that chitosan films without essential oils did not present antimicrobial capacity when they were tested on an agar surface. The authors concluded that possibly the initial counts of inoculum were too high or that no dissolution of chitosan took place.

Nevertheless, the latter reason is in contrast with another recent study about the molecular structure of chitosan acetate films studied by ATR-FTIR spectroscopy upon direct contact with agar plates. This work demonstrated that since the agar plate has very high water activity a considerable release of the carboxylate groups took place by capillarity immediately upon direct contact with the nutrient agar plate (Lagaron et al., 2007). This study also stated that because of the presence of moisture or wet conditions found in many foods that the biocide groups of chitosan, such as protonated glucosamine polymer chains, will be rapidly released from the film and will generate an immediate biocide effect at the surface and possibly underneath the surface, depending on the ease of diffusion provided by the food matrix (Lagaron et al., 2007).

Previous research has been proposed that chitosan can be a suitable antimicrobial agent when coated on an appropriate film. However these results demonstrate that chitosan can only act by contact in the presence of free water. Also, dissociation and diffusion of chitosan from coated films did not occur by reducing the molecular weight of chitosan.

CONCLUSIONS

- The gamma irradiation of chitosan caused a reduction in molecular weight of chitosan.

- Lower concentrations of chitosan solutions (0.5% and 1.0%) and irradiation dosages over 10 kGy showed no inhibition zones against *L. innocua* even at higher concentrations of chitosan (2%). The MW of chitosan solutions irradiated at 10 kGy was 22.4 kDa, and chitosans below this MW were not effective inhibitors of *L. innocua*.

- Previous studies (Feng et al., 2007) have shown that LMW chitosan (2-6 kDa) were more effective antioxidants than native chitosan but this study showed that antimicrobial activity could be lost when MW is less then 22.4 kDa..

- Chitosan as well as LMW chitosan was coated successfully on corona treated multilayer film (2.62 mg/in^2) but showed no antimicrobial inhibition zones against *L. innocua*. LMW chitosan did not diffuse into the surrounding TSA medium as demonstrated by no zones of inhibition around the films. Also, it appeared that growth occurred under the films indicating bacterial growth was not affected by direct contact with chitosan coated films.

- Similar results were obtained with chitosan and LMW chitosan coated paper disks in that no zones of inhibition were observed around the paper disks. These results showed that even LMW chitosan was tightly bound to both film and paper substrates and was not released into the surrounding media. However, paper disks dipped in chitosan solutions but undried (i.e. wet paper disks) showed zones of inhibition. It can be concluded that neither native chitosan nor LMW chitosan can be used as an effective antimicrobial coating on packaging materials unless the food product in contact with the coated surface has sufficient free water or possibly acidity to desorb the chitosan.

APPENDICES

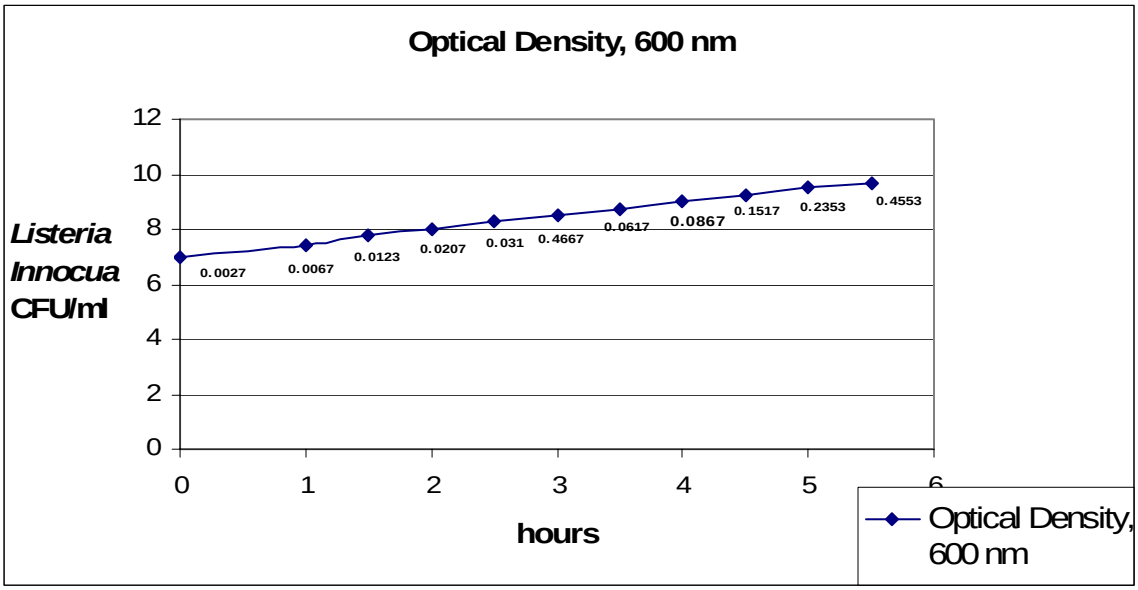
APPENDIX A

Growth Curve for *Listeria innocua*

Population growth of *Listeria innocua* was calculated through the analysis of the turbidity of the stock culture solution. After daily transfer for routine stock (*Listeria innocua*) maintenance, measurements of light absorbency were taken using a Spectroscometer 20 D+ (Thermo spectronic). After each light absorbency measurement, the *Listeria innocua* population was established trough spread plating dilutions of the culture. A relation between light absorbency and culture population was established. By confirming a population at a specific light absorbency reading, a dilute of culture stock could be made to obtain desired population concentrations (CFU/ml).

First a blank of BHI broth growth media was used to zero the Spec 20. After the transfer of 4 ml Listeria innocua, the tube was placed in the gyrotory water bath shaker at 37°C for 4 hours. Then, starting with 0 hour each second hours until hour 12, the light absorbency of the solution was measured. After the light absorbency was measured, 1 ml was removed from the test tube and used to make serial dilutions. The dilutions were plated in triplicate to establish the population of the growing curve. CFU/ml were determined and reported as log CFU/ml. Figure A-1 shows the averaged growth curve of Listeria innocua and the associated averaged Optical density measurement points. This entire experiment was made in triplicates.

Figure A-1: Growth curve of *Listeria innocua* in BHI broth stored at 37°C with constant agitation



APPENDIX B

Statistical Analysis (Duncan's Multiple Range Test)

OUTPUT ordered in kGy

Controll, Chitosan, 1 kGy, 3 kGy, 5 kGy, 10 kGy 1

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The GLM Procedure

Class Level Information

Class	Levels	Values
group	6	A B C D E F

Number of Observations Read 24
Number of Observations Used 24

Controll, Chitosan, 1 kGy, 3 kGy, 5 kGy, 10 kGy 2

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The GLM Procedure

Dependent Variable: a

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	1.00674593	0.20134919	0.96	0.4690
Error	18	3.78449033	0.21024946		
Corrected Total	23	4.79123625			

R-Square	Coeff Var	Root MSE	a Mean
0.210122	8.001142	0.458530	5.730803

Source	DF	Type I SS	Mean Square	F Value	Pr > F
group	5	1.00674593	0.20134919	0.96	0.4690

Source	DF	Type III SS	Mean Square	F Value	Pr > F
group	5	1.00674593	0.20134919	0.96	0.4690

Controll, Chitosan, 1 kGy, 3 kGy, 5 kGy, 10 kGy

3

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The GLM Procedure

Dependent Variable: b

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	20.17415951	4.03483190	18.32	<.0001
Error	18	3.96464847	0.22025825		
Corrected Total	23	24.13880798			

R-Square	Coeff Var	Root MSE	b Mean
0.835756	10.28913	0.469317	4.561285

Source	DF	Type I SS	Mean Square	F Value	Pr > F
group	5	20.17415951	4.03483190	18.32	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
group	5	20.17415951	4.03483190	18.32	<.0001

Controll, Chitosan, 1 kGy, 3 kGy, 5 kGy, 10 kGy

4

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The GLM Procedure

Dependent Variable: c

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	41.99230371	8.39846074	41.69	<.0001
Error	18	3.62570073	0.20142782		
Corrected Total	23	45.61800444			

R-Square	Coeff Var	Root MSE	c Mean
0.920520	9.494631	0.448807	4.726957

Source	DF	Type I SS	Mean Square	F Value	Pr > F
group	5	41.99230371	8.39846074	41.69	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
group	5	41.99230371	8.39846074	41.69	<.0001

The GLM Procedure

Dependent Variable: d

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	72.70632595	14.54126519	21.36	<.0001
Error	18	12.25659848	0.68092214		
Corrected Total	23	84.96292443			

R-Square	Coeff Var	Root MSE	d Mean
0.855742	15.50551	0.825180	5.321852

Source	DF	Type I SS	Mean Square	F Value	Pr > F
group	5	72.70632595	14.54126519	21.36	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
group	5	72.70632595	14.54126519	21.36	<.0001

The GLM Procedure

Dependent Variable: e

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	60.62259080	12.12451816	17.34	<.0001
Error	18	12.58769258	0.69931625		
Corrected Total	23	73.21028338			

R-Square	Coeff Var	Root MSE	e Mean
0.828061	12.65138	0.836251	6.609963

Source	DF	Type I SS	Mean Square	F Value	Pr > F
group	5	60.62259080	12.12451816	17.34	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
group	5	60.62259080	12.12451816	17.34	<.0001

Controll, Chitosan, 1 kGy, 3 kGy, 5 kGy, 10 kGy

The GLM Procedure

Dependent Variable: f

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	28.34579027	5.66915805	5.38	0.0034
Error	18	18.95690370	1.05316132		
Corrected Total	23	47.30269397			

R-Square	Coeff Var	Root MSE	f Mean
0.599243	12.93691	1.026236	7.932627

Source	DF	Type I SS	Mean Square	F Value	Pr > F
group	5	28.34579027	5.66915805	5.38	0.0034

Source	DF	Type III SS	Mean Square	F Value	Pr > F
group	5	28.34579027	5.66915805	5.38	0.0034

The GLM Procedure

Duncan's Multiple Range Test for a

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.210249

Number of Means	2	3	4	5	6
Critical Range	.6812	.7147	.7359	.7505	.7612

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	6.0879	4	A
A			
A	5.8189	4	E
A			
A	5.7745	4	F
A			
A	5.7196	4	D
A			
A	5.5024	4	C
A			
A	5.4815	4	B

The GLM Procedure

Duncan's Multiple Range Test for b

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise

error
rate.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.220258

Number of Means	2	3	4	5	6
Critical Range	.6972	.7315	.7532	.7682	.7791

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	group
	A	6.5393	4	A
	B	4.6277	4	F
	B			
C	B	4.1842	4	D
C	B			
C	B	4.1804	4	E
C	B			
C	B	3.9944	4	C
C				
C		3.8417	4	B

Controll, Chitosan, 1 kGy, 3 kGy, 5 kGy, 10 kGy

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The GLM Procedure

Duncan's Multiple Range Test for c

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error
rate.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.201428

Number of Means	2	3	4	5	6
Critical Range	.6667	.6996	.7203	.7346	.7451

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	7.6549	4	A
B	4.4970	4	F
B			
B	4.1416	4	E
B			
B	4.1355	4	D
B			
B	4.0736	4	C
B			
B	3.8592	4	B

Controll, Chitosan, 1 kGy, 3 kGy, 5 kGy, 10 kGy

22:38 Monday, October 20, 2008

The GLM Procedure

Duncan's Multiple Range Test for d

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.680922

Number of Means	2	3	4	5	6
Critical Range	1.226	1.286	1.324	1.351	1.370

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	9.2018	4	A
B	4.7696	4	F
B			
B	4.6300	4	D
B			
B	4.5644	4	C
B			
B	4.4002	4	E
B			
B	4.3652	4	B

The GLM Procedure

Duncan's Multiple Range Test for e

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	0.699316

Number of Means	2	3	4	5	6
Critical Range	1.242	1.303	1.342	1.369	1.388

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	10.1115	4	A
B	6.3522	4	F
B			
B	6.1610	4	D
B			
B	5.8008	4	C
B			
B	5.6324	4	E
B			
B	5.6017	4	B

The GLM Procedure

Duncan's Multiple Range Test for f

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	18
Error Mean Square	1.053161

Number of Means	2	3	4	5	6
Critical Range	1.525	1.600	1.647	1.680	1.704

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	group
A	10.2017	4	A
B	8.1987	4	F
B			
B	7.5653	4	C
B			
B	7.5247	4	B
B			
B	7.1449	4	D
B			
B	6.9604	4	E

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