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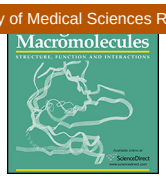

Review

Evaluation of different factors affecting antimicrobial properties of chitosan

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ABSTRACT

Chitosan as one of the natural biopolymers with antimicrobial activities could be a good choice to be applied in many areas including pharmaceuticals, foods, cosmetics, chemicals, agricultural crops, etc. There have been many studies in the literature which show this superb polymer is dependent on many factors to display its antimicrobial properties including the environmental conditions such as pH, type of microorganism, and neighbouring components; and its structural conditions such as molecular weight, degree of deacetylation, derivative form, its concentration, and original source. In this review, after a brief explanation of antimicrobial activity of chitosan and its importance, we will discuss the factors affecting the antimicrobial properties of this biopolymer based on recent studies.

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Contents

1. Introduction

According to the Center for Disease Control and Prevention (CDC), each year in the United States 48 million people get sick, 128,000 are hospitalized, and 3000 die due to foodborne diseases. Therefore, ensuring microbiological safety of food products, while maintaining their nutritional and organoleptic properties, is still a priority nowadays [74]. One option is to use packaging to provide an increased margin of safety and quality. It is so feasible that, the next generation of food packaging will have antimicrobial properties [5,31,45,69,80]. Antimicrobial packaging is considered as one type of active packaging [38,35]. Incorporation of antimicrobial agents into packaging can create an environment inside the package which may delay or prevent the growth of microorganisms on the product's surface and, hence, lead to an extension of its shelf life [8,71]. Antibacterial materials could be classified into two groups; inorganic and organic materials. Inorganics are metals, metal oxides and metal phosphates [90]. Among the inor-

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ganic materials, metal oxides such as TiO_2, ZnO, MgO and CaO are of particular interest as they are not only stable under harsh process conditions, but also generally regarded as safe materials to human beings and animals [85]. Organics are phenols, halogenated compounds, and quaternary ammonium salts; in recent years, studies about antibacterial materials have been focused on natural materials, such as chitosan (CTS) and chitin [25,76,44,17,18,90]. Chitosan has been proved to be non-toxic, biodegradable and biocompatible. Chitosan is approved Generally Recognized as Safe (GRAS) by the [91] and it has a broad-spectrum antimicrobial activity against both gram-positive, and gram-negative bacteria as well as fungi [37,65].

2. Identification of chitin and chitosan

Chitin is a linear polymer of <beta>1,4-linked N-acetyl-D-glucosamine, which looks like <beta>1,4-D-anhydroglucopyranose chain of cellulose except for the acetamide group at C-2 position of anhydroglucopyranoside residue. Similarly to cellulose, native chitin occurs in fibrous crystalline states, i.e. microfibrils [61]. This natural biopolymer (chitin) can be presented in different structural forms, according to its biological function and its natural source; these forms are differentiated according to the arrangement of the carbohydrate chains. The α form has chains arranged alternately antiparallel; the β form has all chains in parallel and the γ-chitin has two chains in one direction with an additional inverted chain [22]. In other words, α-chitin has a rhombic structure with two symmetrical, alternately antiparallel chitin chains. The carbonyl and hydroxyl groups in α-type chitin participate in an increased degree of intermolecular hydrogen bonding, leading to a more compact and stable structure. Therefore, α-type chitin is less likely to swell in water. β-type chitin is monoclinic with two parallel chitin chains, and fewer hydrogen bonds exist between the molecules; hence, it is more loosely structured [82]. Although both allomorphs of chitin (α and β-type) are insoluble in aqueous and common organic solvents, β-chitin displays higher reactivity, swelling and solubility as compared to α-chitin, but as this latter is more widely spread in the biomass, mainly as a major component of the crustaceans shells, it is preferentially used in industries and in research laboratories [4].

Also, chitin can be found with varying degrees of acetylation (DA), ranging from fully acetylated to tollally deacetylated. The degree of acetylation is very important because of its effects on physical properties of chitin. For example, as the degree of acetylation increases, the degree of solubility in solvents decreases [84].

On the other hand, chitosan is a high molecular weight cationic polysaccharide consisting of (1-4)-2-amino-2-deoxy<beta>D-glucan [104], and usually refers to a family of chitin derivatives obtained after partial deacetylation [103]. The degree of acetylation of chitosan is characterized by the molar fraction of N-acetylated units (DA) or as a percentage of acetylation (DA%). When the degree of acetylation is lower than 0.5 (50%), chitosan becomes soluble in acidic aqueous solutions due to the protonation of NH_2 group at the C-2 position of glucosamine units [103,87]. The deacetylation of chitin to produce chitosan is generally accomplished using three different methods: chemical (hot alkaline), microbial, and enzyme-based methods. In the enzymatic and microbiological methods, enzymes and microorganisms, respectively, deacetylate chitin [82]. Chitosan, obtained from the alkaline deacetylation of chitin, is a functional polysaccharide with great potential in food applications and packaging requirements. It is the most investigated polysaccharide for antimicrobial edible films and coatings development due to its inherent antimicrobial and antifungal properties and film forming ability [25]. Studies have purposed that cultivation of selected fungi could provide an effective source of chitosan for industrial applications; e.g., efficacy of chitosan from *Mucor rouxii* in inhibiting *Listeria monocytogenes* in bovine meat pate [3]. [34] reported that chitosan is extractable not only from *Zygomycetes* fungi, but also from non-*Zygomycetes* fungi.

3. Antimicrobial properties of chitosan

Chitosan contains three types of reactive functional groups, an amino group on C-6 position as well as both primary and secondary hydroxyl groups at the C-6, C-3 positions, respectively. The amino contents are the main reason for the difference between chitin and chitosan structures and their physicochemical properties which are correlated with their chelation, flocculation and biological functions [93,94]. Chitosan also represents interesting properties such as excellent film forming capacities and gas and aroma barrier properties at dry condition, which makes it a suitable material for designing food coatings and packaging structures [7,88]. Chitosan inhibits the growth of a wide variety of fungi, yeasts, and bacteria [47,104,73,25,1,100,88], although chitosan activity against fungi has been shown to be less efficient as compared with its activity against bacteria [107,89]. Chitosan is a weak base and is insoluble in water and organic solvents. However, it is soluble in dilute aqueous acidic solutions (pH < 6.5), which can convert glucosamine units into soluble form $R\text{-}NH_3^+$ [43,66] however, chitosan shows its antibacterial activity only in an acidic medium, which is usually ascribed to the poor solubility of chitosan at high pH [54,55,59,66].

Antimicrobial effect of chitosan and its influence on shelf life of different foods has been investigated in many studies which are summarized in Table 1.

Several mechanisms have been proposed for the antimicrobial activity by chitosan (Fig. 1) including:

a positively charged chitosan molecules interfere with the negatively charged residues on the bacterial surface. Chitosan interacts with the membrane of the bacteria to alter cell permeability [73,11,52,96,97]. Li et al. [52] demonstrated that the antibacterial mechanism of derivative of chitosan (O-quaternary ammonium N-acyl thiourea chitosan) was due to the interactions of cationic NH^{3+} groups with negative charged cell membranes which consequently increased the membrane permeability and membrane lysis (Fig. 2). In the absence of chitosan derivative, the transmission electron micrograph of *Escherichia coli* indicated that normal cells were surrounded by the cell membranes with compact surface, without release of intracellular components and notable ruptures on the cell surfaces (Fig. 2a). The damaging impact on bacteria by derivative of chitosan can be observed from the change in the bacterial morphology. Fig. 2b gives the TEM images of *E. coli* and shows how the treated bacteria turned into irregular bacterial cell with broken wall and the cell contents infiltrated.

b the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis [104,10,19,21,77];

c chitosan also inhibits the microbial growth by the chelation of nutrients and essential metals[104,11,50,51,16];

d chitosan on the surface of the cell can form a polymer membrane which prevents nutrients from entering the cell [21,30,54,55,105] or acts as an oxygen barrier which can inhibit the growth of aerobic bacteria [104,19].

4. Factors influencing the antimicrobial activity of chitosan

During the synthesis and use of chitosan, attention should be paid to the factors which influence activity of chitosan (Fig. 3).

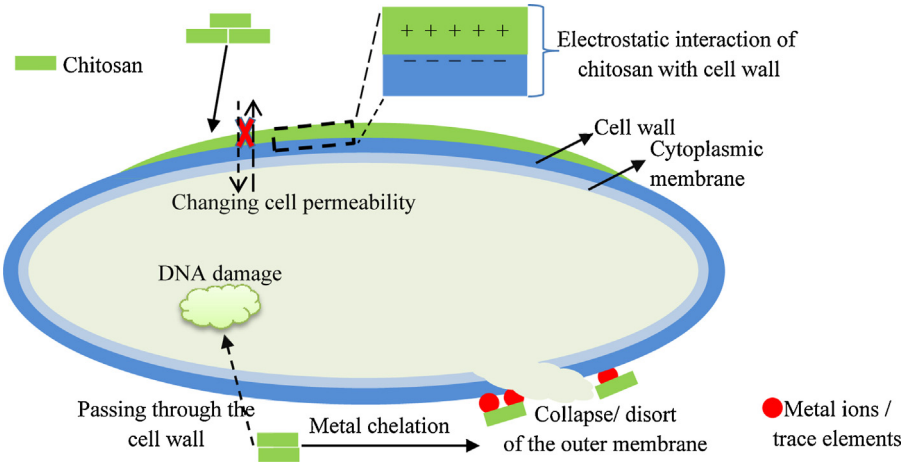

Fig. 1. Schematic representation of antimicrobial mechanisms of chitosan and its derivatives.

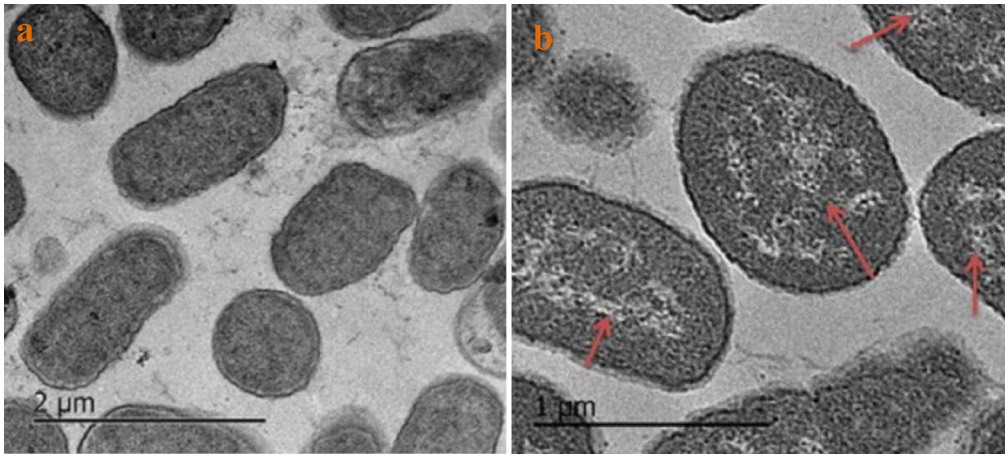

Fig. 2. TEM images of *E. coli*: (a) native and untreated cells; and (b) after treatment with chitosan (Data from Ref. [51]).

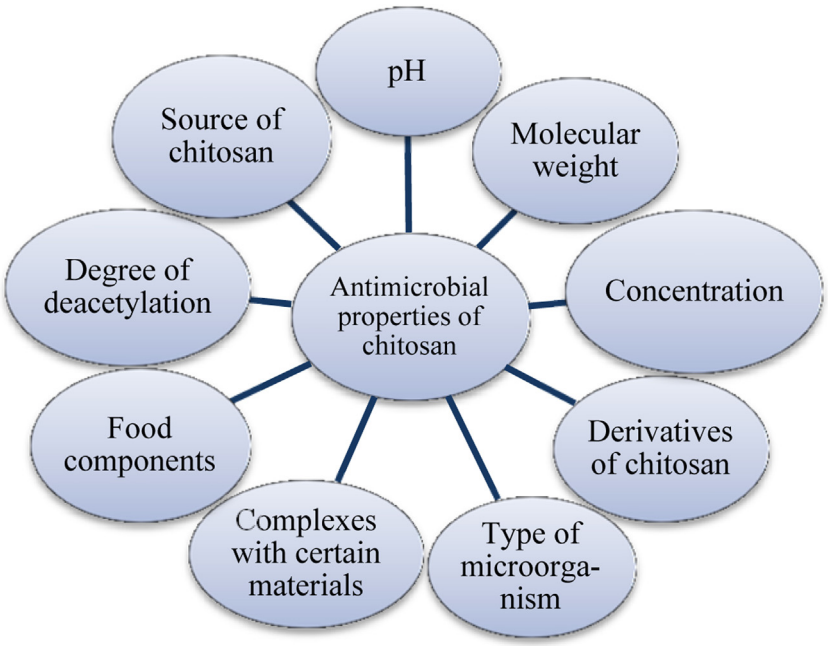

Fig. 3. Different factors affecting antimicrobial properties of chitosan.

Table 1
Selected studies evaluating antimicrobial effects of chitosan on different foods.

Application and preparation methods	Tested foods	Target microorganism	References
Coating with 2% chitosan and/or a mixture having 2% chitosan and 1.5% clove oil	Cooked pork sausages	Total viable count, Psychrotrophic bacteria count	[47]
Combined effect of chitosan and pomegranate peel extract	Pacific white shrimp	Total aerobic plate counts	[104]
Combined effect of chitosan (1% (w/v)), carvacrol nanoemulsion 0.05% (w/v), gamma irradiation (0.25 kGy) and modified atmosphere packaging	Green beans	*Escherichia coli* O157:H7, *Salmonella Typhimurium*	[73]
Fruits immersed in chitosan solutions (amount of chitosan applied per unit area was $10gm^{-2}$)	Papaya fruits	Mesophilic bacteria, Yeasts and molds	[20]
Chitosan solution (1 mg/g) and combined effect of chitosan and oregano essential oil (EO) (1 mg/g chitosan + 3 μl/g oregano EO)	Cured chicken meat	Lactic acid bacteria, *Enterobacteriacae*, *E. coli* O157:H7, *Listeria monocytogenes*, Psychrophilic, Aerobic plate count	[76]
Lemongrass essential oil at concentrations of 0.5% and 1.0% incorporated into 0.5% and 1.0% chitosan solution	Bell pepper	*Anthracnose*	[1]
Chitosan solution 1.5% (w/v) and chlorine dioxide + chitosan coating (28 mg L^{-1} + 1.5% (w/v))	Fresh-cut bamboo shoots	Total aerobic bacteria, Yeast and mould	[100]
LDPE/chitosan composite film (embedding 1, 3 and 5% chitosan (w/w) in low density polyethylene matrix)	Chilled fish	*Escherichia coli*	[67]
Chitosan–nanocellulose biocomposites	Ground meat	*lactic acid bacteria*	[18]
Combined effect of chitosan solution (1 g/100 ml) and modified atmosphere packaging (70% CO_2, 30% N_2)	Chicken breast fillets	*Pseudomonas* spp., *Lactic Acid Bacteria*, *Enterobacteriaceae*	[46]
Combined effect of carvacrol, bergamot, lemon, mandarin essential oils (EO) and chitosan (concentration of EO in the coating formulations was 0.05% (w/v)	Broccoli florets	*Listeria monocytogenes*	[74]
Meat samples coated with 2 g/100 ml chitosan	Ready-to-cook meat products	*Bacillus cereus*, *E. coli*, *Staphylococcus aureus*, *Pseudomonas fluorescens*	[37]
Aqueous suspension of chitosan (5 mg chitosan/g pâté)	Bovine meat pâté	*Listeria monocytogenes*	[3]
Chitosan–/poly(vinyl alcohol solution (glutaraldehyde as the cross-linker)	Tomato	*Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*	[88]
Chitosan/methyl cellulose and chitosan/methyl cellulose film incorporating vanillin	Fresh-cut cantaloupe and pineapple	*Escherichia coli*, *Saccharomyces cerevisiae*	[71]
Chitosan-coated plastic film alone or incorporating sodium lactate, potassium sorbate, sodium benzoate, nisin and sodium diacetate	Ham steaks and cold-smoked salmon	*Listeria monocytogenes*	[102]
Chitosan glutamate (0.1 to 5 g/l)	Apple juice	*Zygosaccharomyces bailii*, *Saccharomyces exiguous*, *S. cerevisiae*, *Saccharomycodes ludwigii*, *Schizosaccharomyces pombe*	[68]

4.1. Effect of pH

Chitosan is polycationic at pH < 6 and interacts readily with negatively charged substances such as proteins, anionic polysaccharides, fatty acids, bile acids and phospholipids due to the high density of amino groups present on the polymer [39,58,68]. The mechanism of the antimicrobial activity of chitosan and its derivatives is unknown. It has been suggested that a positive charge on the NH_3^+ group of the glucosamine monomer at pH < 6.3 allows interactions with negatively charged microbial cell membranes which leads to the leakage of intracellular constituents [30,54,55,77]. Antimicrobial activity of chitosan is higher at low pH; this is due to the fact that the amino groups of chitosan become ionized at pH below 6 [19,92]. In a study by Younes et al. [103], it has been shown that with reducing pH, chitosan adsorption on bacterial cells will be increased probably due to increase of chitosan positive charge.

But at higher pH (>6), chitosan tends to lose its charge and may precipitate from solution due to deprotonation of the amino groups [32,98,29,15]. Kulikov et al. [41] reported that antibacterial activity of chitosan against *Klebsiella pneumoniae* was closely associated with its polycationic nature, and depended on the degree of protonation of the chitosan amino groups, which, in turn was the function of the degree of polymerization and the pH values of the medium. Devlieghere et al. [19] showed that native chitosan was significantly more active against *Candida lambica* at pH 4.0 than at pH 6.0. Also, it was demonstrated by Roller and Covill [68] that inhibitory properties of chitosan against *Mucor racemosus* were greater at lower pH. In another study, antimicrobial capacity of chitosonium acetate films against the growth of *Staphylococcus aureus* in pH 6.2 and pH 7.4 was investigated by Fernandez-Saiz et al. [24]. Their tests carried out at a lower pH, i.e. 6.2, showed less bacterial counts indicating a stronger biocidic effect; this finding is related to the particular pK_a of this biopolymer (i.e. 6.4), which is near the pH 6.2 value. At this pH, the amount of positively charged amino groups (active groups) is close to 75% in chitosan while at pH of 7.4, this quantity drops until approximately 10%.

4.2. Molecular weight of chitosan

Chitosans can be distinguished by their molecular weight (MW): high molecular weight (HMW) chitosan, low molecular weight (LMW) chitosan, and oligochitosan (short chain chitosan) [41]. As decribed in Table 2, numerous studies have reported a correlation between bactericidal activity of chitosan and its molecular weight. HMW chitosan can not pass through the microbial membrane and hence stack on the cell surface, which blocks nutrient transport into the microbial cell membrane, resulting in cell lysis [50,51,86,16]. On the other hand, dissociated chitosan molecules in solution, with lower molecular weight (<5000 kDa), could bind with

Table 2
Selected studies evaluating the effect of molecular weight on antimicrobial activity of chitosan.

Molecular weight of chitosan and preparation methods	Target microorganism	Major findings	References
Chitosan solution with molecular weight 150 and 300 kDa	Mesophilic bacteria, Yeasts and molds	- 150 kDa chitosan solution was more adequate to preserve the papaya fruits - For 150 kDa chitosan, the log CFU/g of mesophilic bacteria and yeasts and molds were, respectively, 1.3 and 2 times lower	[20]
Carvacrol incorporated chitosan edible films, (chitosan extracted from shrimp shell with MW180 and 400 kDa)	*Pseudomona fragi*, *Shewanella putrefaciens*, *Aeromonas hydrophila*	- Chitosan with higher MW formed film forming dispersions with higher viscosity and higher particle size than those formed from chitosan with lower MW -*P. fragi* was the most resistant strain to the antimicrobial effect of films, while *Sh. putrefaciens* was the most sensitive - It seems that the higher the MW, the higher the inhibition effect on the specific studied strains	[107]
Chitosan solution, (3.3, 7.1, 29.2, 72.1, 156, 300 kDa)	*Escherichia coli*, *Staphylococcus aureus*	-In acidic pH conditions, chitosan activity increased with increasing MW, irrespective of the temperature and bacteria tested - By contrast, at pH 7.0, chitosans with MW >29.2 kDa greatly lost their activity, whereas the activities of the smaller chitosans (29.2, 7.7, and 3.3 kDa) increased as the MW decreased	[9]
Oligochitosan solution, molecular weights 0.73, 1.52, 2.09, 5.98, 8.39, 9.69, 15.06, 19.99, 70 and 600 kDa	*Candida* species and clinical isolates of *C. albicans*	- Oligochitosans showed a high fungistatic activity (MIC 8–512 μg/ml) - Oligochitosans with MW between 10 and 20 kDa displayed maximal activity in suppressing yeast cells multiplication and caused severe cell wall alteration	[41]
Chitosan solution 20 cp (low molecular weight chitosan) and 40 cp (high molecular weight chitosan) with different concentrations (0.25%, 0.5% and 1%)	Aerobic mesophilic bacteria	- Low molecular weight chitosan best reduces total viable counts compared with high molecular weight chitosan	[70]
Chitosan solution, molecular weights (0.5×10^4, 3.7×10^4, 5.7×10^4 and 2.9×10^5 g/mol), concentrations (500, 1000, 2000 and 4000 mg/L)	*Botrytis cinerea* (gray mold)	- Chitosan with a molecular weight of 5.7×10^4 g/mol provided an excellent control of gray mold among all different molecular weight chitosans - Chitosan of 0.5×10^4 g/mol was the least active one at low concentrations (500 and 1000 mg/L) in the in vivo experiments	[2]

DNA and inhibit synthesis of mRNA through penetration toward the nuclei of the microorganisms [42]; [11]; [40]; [77]. While in HMW form, the dissociated chitosan molecules could interact with the membrane of the cell to alter cell permeability [2,40,48,57]. Ye et al. [101] reported that LMW chitosan-coated film was more effective against *L. monocytogenes* than the medium MW chitosan-coated film. Inhibition activity of chitosan solution with MW of 3, 50, and 1000 kDa against *E.coli* was also evaluated by Li et al. [51]. Their results demonstrated in general, chitosan inhibited the growth of *E. coli*, but chitosan of 50 kDa showed the most effective inhibition activity. No et al. [60] revealed that 0.1% chitosan (MW = 1671, 1106, 746, 470, 224 and 28 kDa) showed stronger bactericidal effects against gram-positive bacteria than gram-negative bacteria. For gram-negative bacteria, chitosan of 746 kDa appeared most effective against *E. coli* and *Pseudomonas fluorescens*, compared with chitosan 470 kDa against *Salmonella Typhimurium* and *Vibrio parahaemolyticus*. Chitosan of MW = 1106 and 224 kDa possessed weak or no antibacterial activity (MW = 28 kDa) against *S. typhimurium*. In contrast to the response of gram-negative bacteria, growth of gram-positive bacteria was almost or completely suppressed by widely different MW chitosans.

4.3. Concentration of chitosan

At lower concentrations, chitosan binds to the negatively charged cell surface, disturbs the cell membrane, and causes death of the cell by inducing leakage of intracellular components. Whereas, at higher concentrations, the protonated chitosan may coat the cell surface and prevent the leakage of intracellular components. In addition, the positively charged bacterial cells repel each other and prevent agglutination [53]. An antimicrobial packaging material was prepared by uniformly embedding 1, 3 and 5% chitosan (w/w) in low density polyethylene (LDPE) matrix by [67]. The antimicrobial assay against *E. coli* proved that LDPE/chitosan composite (LDPE/CS) films were highly efficient than virgin LDPE films. Virgin LDPE and 1%, 3% and 5% LDPE/CS films tested as packaging films for chill stored tilapia showed that samples packed in LDPE films were rejected by 7th day whereas fish packed in 1%, 3% were remained acceptable up to 15 days. This study revealed that 3% LDPE/CS films had a better physical and antimicrobial property and enhanced the keeping quality of Tilapia steaks during chilled storage when compared to the other films. In another study by [56], different molecular weight chitosans (5.5×10^4–15.5×10^4 Da) in various concentrations (20, 50, 100, 200, 500 and 1000 ppm) were used to evaluate the effects of the MW and concentration of chitosan against *E. coli*. Their results showed that all chitosan samples with MW from 5.5×10^4–15.5×10^4 Da had good antimicrobial activities at high concentrations (over 200 ppm), and all samples at low concentration (20 ppm) could promote the growth of *E. coli*. They suggested that mechanism of chitosan antibacterial activity could be described by the fact that it could make the bacteria flocculate and kills them, but at low concentration (20 ppm), chitosan could not flocculate and kill all the bacteria in the culture medium and the survival would go on by reproducing. Antimicrobial activity of the cotton fabrics treated with different chitosan concentrations reported by [21] indicated that maximum antimicrobial activity was obtained when the cotton fabrics were treated with 0.5–0.75% chitosan with molecular weight of 1.5–5 kDa and increasing chitosan concentration to 1% leads to a decrement in the antimicrobial activity.

4.4. Complexes of chitosan with certain materials

In order to improve antimicrobial activity, complexes of chitosan with certain materials can be prepared. Incorporation of essential oils (EOs) in chitosan based coatings has gained interest in the agricultural sciences owing to the bactericidal and fungicidal properties associated with these volatile compounds [1]. Recently, different EOs, such as clove, carvacrol, oregano and lemongrass, have been successfully incorporated into chitosan showing strong antimicrobial activity against a wide range of microorganisms [47,73,76,25]. Also Ojagh et al. [62] showed that a unique compatibility can be achieved between chitosan and cinnamon EOs; their incorporation improved the antibacterial properties of chitosan. Films containing Cinnamon EOs are useful for coating of highly perishable foods such as fish and poultry. In a further experiment by Gómez-Estaca et al. [28], a complex of gelatin–chitosan film incorporating clove EOs was applied to fish during chilled storage. Results of this study revealed that clove film delayed or even prevented both the growth of microorganisms and the occurrence of total volatile nitrogen. Therefore film incorporating clove EOs could assure an extended shelf-life for chill-stored fish. [76] demonstrated the synergistic antibacterial effects of chitosan and oregano EOs against spoilage inducers in chicken meat stored at 3 and 8 °C.

Chitosan macromolecule contains great deal of amine and hydroxyl groups which give it the ability to form metal complexes. Generally, the structure of chitosan-metal complexes depends on chitosan/metal ion molar ratio, type of metal ion, molecular weight and deacetylation of chitosan as well as the preparation conditions [31]. Sanpui et al. [72] reported that presence of a small percentage (2.15% w/w) of metal nanoparticles in chitosan-Ag-nanoparticle composite was enough to significantly enhance inactivation of *E.coli* as compared with unaltered chitosan. On the other hand, chitosan helps stabilize the silver nanoparticles (AgNP) and prevents AgNP agglomeration below a critical concentration. Chitosan also confers a positive charge to the surface of nanoparticles, enhancing their binding to the negative charges present in the cell wall [44]. Chitosan/Ag/ZnO blend films were also prepared via the method of sol-cast transformation by Li et al. [51]. Test of antimicrobial activities showed that CTS/Ag/ZnO blend films had higher antimicrobial activities than CTS/Ag and CTS/ZnO blend films, indicating that the composite of Ag and ZnO enhanced the antimicrobial activities of chitosan. In another work, Ye et al. [102] assessed the effect of chitosan-coated plastic films incorporating five GRAS antimicrobials including nisin, sodium lactate (SL), sodium diacetate (SD), potassium sorbate (PS), and sodium benzoate (SB) against *L. monocytogenes* on cold-smoked salmon. Results of this study showed that film incorporating SL was the most effective, completely inhibiting the growth of *L. monocytogenes* during 10 days of storage. *L. monocytogenes* in samples packaged within other four antimicrobial films grew, but the increase in counts was lower than two control films: plain and chitosan-coated films. Yang et al. [100] investigated the effects of aqueous chlorine dioxide (ClO_2) combined with chitosan coating on microbial growth and quality maintenance of fresh-cut bamboo shoots during cold storage. Their results indicated that treatment with 28 mg L^{-1} ClO_2 plus chitosan coating inhibited the increase in respiration rate and firmness, delayed enzymatic browning and reduced microorganism counts of total aerobic bacteria and yeasts and moulds compared with control treatment. The efficiency was better than that of ClO_2 or chitosan treatment alone.

4.5. Derivatives of chitosan

Due to its unique polycationic nature, chitosan and its derivatives have been recommended for applications in agriculture, food, biomedical, biotechnology and pharmaceutical fields [23,75,99]. However, the antibacterial functions of chitosan are limited because amino groups on chitosan backbone can only function as relatively weak positive charge centers. To improve the antimicrobial activity of chitosan, it is reasonable to enhance the strength of positive charges on the chitosan molecules by endowing it with some more positively charged groups [95]. Therefore, in the past two decades, extensive investigations have been carried out to increase solubility of chitosan in water and broaden its applications by preparing functional derivatives of chitosan such as carboxymethyl chitosan and quaternized carboxymethyl chitosan [79], chitosan-N-arginine by reacting amino groups of chitosan with arginine [95], N-alkylated disaccharide chitosan [98], water-soluble maltose chitosan derivative [99], water-soluble quaternary chitosan derivatives obtained by N-acylation with betaine [32], and water-soluble oligochitosans [42,41].

Antimicrobial effect of chitosan derivatives has been investigated in many studies. For example, antimicrobial behaviors of chitosan and its derivatives against species of bacteria (*E. coli*, *S. aureus, Pseudomonas aeruginosa* and Sarcina) and four crop-threatening pathogenic fungi (*Alternaria solani*, *Fusarium oxysoporum* f. sp. Vasinfectum, *Colletotrichum gloesporioides* Penz and *Phyllisticta sp. Zingiberi*) were investigated by Zhong et al. [106]. Their results indicated that antimicrobial activities of acetyl phenyl-thiosemicarbazone derivatives were much better than that of pure chitosan. Comparative antimicrobial activity evaluation of O, N-carboxymethyl chitosan–zinc complex and chitosan–zinc complex was carried out by Patale and Patravale [64] against *S. aureus* and *E. coli*. It was observed that the developed novel O, N-carboxymethyl chitosan–zinc complex exhibited superior antimicrobial activity compared with chitosan–zinc complex.

However, the antimicrobial activity of these materials (with single functional group solubility in water) is still lower than that of the common antimicrobial agents currently used. Therefore, synthesizing new derivatives which contain both antibacterial and water-soluble groups may facilitate the use of chitosan as an antibacterial material [52]. In order to achieve this goal, two types of reactive functional groups present in chitosan contains, i.e., a primary amine and two hydroxyl groups, can be used to chemically alter its properties and ultimately lead to an increased antimicrobial effect of the chitosan; in fact, this type of chitosan is called derivatives with dual-antibacterial functional groups [26]. Li et al. [52] reported that chitosan derivative with double functional groups of *O*-quaternary ammonium N-acyl thiourea chitosan (OQCATUCS) had higher growth suppression against gram-positive and gram-negative bacteria compared with corresponding single functional group and chitosan (OQCATUCS > *O*-quaternary ammonium chitosan > chitosan). Also, OQCATUCS exhibited an excellent solubility over a wide pH range at room temperature (in OQCATUCS, hydroxyl group reacted with ammonium chloride and amino group reacted with chloroacetyl thiourea).

4.6. Type of microorganism

Antibacterial activities of chitosan and its oligomers with different molecular weights were examined by [60] against four gram-negative and seven gram-positive bacteria. It was found that chitosan generally showed stronger bactericidal effects on gram-positive bacteria than gram-negative ones. [24] also obtained similar results when testing antimicrobial capacity of chitosonium acetate films against *S. aureus* and Salmonella. In this study, although the MIC of the films tested corresponded to 40 mg for *S. aureus*, the quantities used in the work did not reach an inhibitory effect against Salmonella. They claimed that their results were in accordance with the hypothesis of an electrostatic interaction between chitosan and the cell wall, that in this sense, gram-positive microorganisms should be more susceptible than gram-negative ones since their wall is composed of a thick peptidoglycan layer

and by polymers called teichoic acids. This teichoic acid backbone is highly charged by phosphate groups with a negative charge, which could establish electrostatic interactions with cationic antimicrobial compounds such as chitosan. Contrary to these results, Devlieghere et al. [19] and Chung et al. (2004) [12] reported that chitosan showed stronger antimicrobial effects for gram-negative bacteria than gram-positive counterparts.

Recently, Younes et al. [103] demonstrated that antibacterial activity was further enhanced for gram-negative bacteria with decreasing MW, whereas, opposite effect was observed with the gram-positive bacteria. Concerning the antifungal activity, the influence of chitosan characteristics was dependent on the particular type of fungus. Fungal growth decreased with increasing MW for *F. oxysporum* and decreasing acetylation degrees (DA) for *A. solani*, but no MW or DA dependences were observed with *Aspergillus niger*. Also, Ziani et al. [107] showed that the antifungal capacity of chitosan does not only depend on the chitosan formulation, but also on the fungus type and on the type of chitosan (films and solutions based on chitosan). For instance solutions also displayed higher efficiency on the growth of *Rhizopus oryzae* but not on that of *A. niger* where the films were found to be more effective.

4.7. Food components (role of inorganic cations)

In addition to previous factors, the antimicrobial activity of chitosan is also very dependent on the food components. Since the charges on chitosan and the concomitantly electrostatic forces are responsible for its antimicrobial activity, each food component that can influence these interactions will inhibit the activity of chitosan [19].

Goldberg et al. [27] found that inorganic cations (Na^+, Mg^{2+}) inhibited the chitosan-mediated adhesion of *E. coli* to hexadecane, presumably by interfering with the electrostatic interactions responsible for adsorption of the polymer onto the bacterial surface. In a study for evaluating the effect of metal salt addition ($MgCl_2$, $CaCl_2$, $BaCl_2$ or NaCl) on the antibacterial activity of water-soluble chitosan derivative by Chung et al. [14], their results revealed that the metal ions reduced the antibacterial activity of chitosan derivative and increased the final viable population of *S. aureus*. It is probably due to functional groups of chitosan (free amino groups) which were chelated with metal ions, and caused a decrease in antibacterial activity of chitosan [13].

Devlieghere et al. [19] reported that starch, whey proteins, and NaCl had a negative effect on the antimicrobial activity of chitosan, while oil conversely had no influence. Chung et al. [13] evaluated the effects of ionic strength and addition of EDTA on the growth of waterborne pathogens (*E. coli* and *S. aureus*) by chitosan at different NaCl concentrations (0, 0.2, 0.4, and 0.6 M) and EDTA (0, 1, 2, 3 mM). They suggested that higher ionic strength (0.6 M NaCl) enhanced the solubility of chitosan and thus increased its antibacterial activity. Also, antibacterial activity of chitosan against *E. coli* was enhanced by EDTA. However, the antibacterial activity of chitosan against *S. aureus* was partially suppressed by EDTA which possibly can be explained by the fact that the carboxyl groups (COO^-) of EDTA react with the amino groups (NH^+_3) of chitosan, which shields the most important antibacterial functional groups of chitosan and leads to a decrease in its antibacterial activity. In another study, Li et al. [49] demonstrated that there was no significant difference in the antibacterial activity of chitosan when the concentration of NaCl increased from 0.5% to 2.0%.

4.8. Degree of deacetylation (DD)

Chitosan is produced commercially by deacetylation of chitin. In the process of deacetylation, acetyl groups from the molecular chain of chitin are removed to form amino groups. The degree of deacetylation, which determines the content of free amino groups in polysaccharides, can be employed to differentiate between chitin and chitosan. It is very well known that the degree of deacetylation is one of the most important chemical characteristics, which could influence the performance of chitosan in many applications [57,83]. Overall, the larger is the DD, the higher is the solubility in acidic conditions. Generally, chitosan with higher DD, which has a higher positive charge, would be expected to have stronger antibacterial activity [87]. Reported by Byun et al. [6] the superior antibacterial activity of g-chitosan (prepared from ground shell with DD 81.56%) over that of e-chitosan (prepared from entire shell with DD 62.71%) was likely due to its higher solubility and DD.

Park et al. [63] evaluated the effect of hetero-chitosans and chitosan oligosacchides on the growth of three gram-negative and five gram-positive bacteria. They found that although the antimicrobial activity of hetero-chitosans and chitosan oligosaccharides was different against the tested bacteria, 25% acetylated chitosan (75% deacetylated chitin) had the highest inhibitory activity as compared with the activity of 10% and 50% acetylated chitosan. In another work, antimicrobial activity of hetero-chitosans and the effect of deacetylation conditions (vacuum, nitrogen and regular atmospheres) on antimicrobial activity of chitosans against *E. coli*, *S. aureus* and *Candida albicans* was investigated by Hongpattarakere and Riyaphan [33]. Their results showed that chitosans obtained under atmospheric conditions exhibited highest antimicrobial activity, due to lower DA, whereas chitosans obtained under nitrogen atmosphere showed the least inhibition against all test microorganisms. In a study by Takahashi et al. [81] the influence of DA of chitosan on inhibiting the growth of *S. aureus* was investigated by two methods: incubation using a mannitol salt agar medium, and a conductimetric assay. Their results showed that in both methods, chitosan with a lower DA successfully inhibited growth of *S. aureus*.

4.9. Sources of chitosan

Chitosan is a natural antimicrobial agent found in the shells of crustaceans, such as crab, shrimp, squid pen, and crawfish [78,60]. Recently, some studies have pointed to the possibility of chitosan production from fungi. In one study, chitosan was extracted from cell wall of filamentous fungus, *R. oryzae* by Jeihanipour et al. [36] and its antimicrobial properties was studied against *E. coli*, *K. pneumoniae* and *S. aureus*. Their resulted showed that antimicrobial activity of fungal chitosan was lower than that of crustacean shells chitosan. But fungal chitosan similar to crustacean shells chitosan exhibited better inhibitory effects against gram-positive bacteria compared with gram-negative ones. Chien et al. [11] reported that crude chitin from crab shells did not show any antimicrobial activity but chitin from shiitake stipes and mushroom exhibited a better inhibitory effect on bacterial growth than chitin from crab shells. After purification, chitosan from shiitake stipes and crab shells exhibited excellent antimicrobial activities against species of pathogenic bacteria. However, chitosan from shiitake stipes was slightly more effective than that from crab shells. It was demonstrated by Byun et al. [6] that chitosans prepared from ground and entire crab leg shell exhibited drastic differences in their physicochemical and functional characteristics. For example, chitosan prepared from ground shell had significantly higher nitrogen content, degree of deacetylation, solubility, viscosity and antibacterial activity than chitosan prepared from entire shell.

Antibacterial activities of oligomer and polymer chitosans from different sources (shrimp, crab and squid) were also examined against foodborne pathogenic bacteria by Sukmark et al. [78]. Based on their results, the source, DD and molecular size of chitosan must be chosen selectively to control the target foodborne pathogens.

5. Conclusion

Chitosan and its derivatives as natural antimicrobial agents can be applied in agriculture, food, biomedical, biotechnology and pharmaceutical fields. However, its antimicrobial activity is dependent on many factors such as its molecular size, source, adjucunt components, pH, concentration, type of microorganism, etc, which should be considered before being applied. It is recommended that to achieve the highest antimicrobial activity, optimum conditions of chitosan application should be investigated and tested.

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