Antimicrobial effects of chitosans and chitooligosaccharides, upon *Staphylococcus aureus* and *Escherichia coli*, in food model systems

João C. Fernandes, Freni K. Tavaria, José C. Soares, Oscar S. Ramos, M. João Monteiro, Manuela E. Pintado, F. Xavier Malcata *

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal

The objective of this study was to elucidate the controversial relationship between the molecular weight (MW) of chitosans and their antibacterial activity (upon different inoculum levels, at several concentrations). The influence of food components on the activity was also ascertained, as well as acceptance by a sensory panel. All the compounds tested exhibited antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. This activity was shown to be closely dependent on the inoculum level, MW and concentration used. Within 4 h at 10^3 cells/mL, all five compounds, at every concentration (0.5%, 0.25% and 0.1%, w/v), proved to be bactericidal; for higher inocula, 0.1% (w/v) was only bacteriostatic; at 10^7 or 10^5 cells/mL, and independently of the inoculum level, 0.25% (w/v) of any chitooligosaccharide (COS) mixture was sufficient to reduce the *E. coli* initial population by at least 3 log cycles; COS never exhibited bactericidal action over *S. aureus*, unlike high and medium MW chitosans—which, at 0.5% (w/v), presented a bactericidal effect even against 10^7 cells/mL. When incorporated in liquid food matrices, medium and high MW chitosans maintained their activity, for both matrices and bacteria, although a slower activity was noticeable in milk; however, COS lost their activity upon both bacteria in milk after 4–8 h. Furthermore, addition of chitosans to apple juice led to several unpleasant off-flavors, such as astringency and after taste—which increased in magnitude with MW.

Introduction

Chitin is a natural polymer, found in the exoskeletons of crustaceans and insects, as well as in the cell walls of certain fungi (Rinaudo, 2006). Full (or partial) deacetylation of chitin produces chitosan—a linear polysaccharide composed mainly of β-1,4-2-deoxy-2-amino-D-glucopyranose, and of β-1,4-2-deoxy-2-acetamido-D-glucopyranose residues to a lesser extent (Vernazza et al., 2005). Evidence has been put forward that chitosan possesses various biological activities, viz. antioxidant (Chien et al., 2007), cholesterol lowering (Koide, 1998), and antibacterial and antifungal properties (Liu et al., 2006; Rhoades and Roller, 2000; Tikhonov et al., 2006; Uchida et al., 1989), besides being useful as an active dietary component for body fat loss (Ormrod et al., 1998; Woodgate and Conquer, 2003). These features, combined with its biocompatibility and biodegradability, make it an interesting polymer for several applications in agriculture (Pospieszny et al., 1994), as well as in pharmaceutical, cosmetic (Kim et al., 2006; Ravi-Kumar et al., 2004), food (Devlieghere et al., 2004) and textile (Lim and Hudson, 2004) industries, and also in medicine (Shi et al., 2006). However, its high-molecular weight (MW)—most commercial chitosans have indeed a MW ranging between 100 and 1200 kDa (Roller and Covill, 1999), has limited its practical applications due to its insolubility at pH values above 6.3 (Okuyama et al., 2000; Seo et al., 2007).

Recent studies have focused on conversion of chitosan to oligosaccharides (termed chitooligosaccharides, COS)—because the latter are not only readily soluble in water due to their shorter chain lengths (generally, the MW of COS is 10 kDa or less) (Kim and Rajapakse, 2005) and free amino groups in D-glucosamine units, but also easily absorbed through the intestine, quickly getting into the blood flow (Chae et al., 2005; Kim and Rajapakse, 2005). The aforementioned properties, in addition to the positive charge of COS (which allows them to bind strongly to negatively charged surfaces), are responsible for many observed biological activities, such as lowering high blood pressure (Giustina and Ventura, 1995), controlling arthritis (Lee et al., 2003), treatment of diabetes mellitus (Liu et al., 2007) and immuno-stimulation (Suzuki et al., 1986), in addition to prebiotic activity (Lee et al., 2003). Furthermore, such oligomers are potentially more advantageous than chitin and chitosan as nutraceutical food additives because, unlike the

* Corresponding author.
  E-mail address: fxmalcata@esb.ucp.pt (F. Xavier Malcata).

former, COS are degraded in the human intestine (Shahidi et al., 1999).

The reported minimum inhibitory concentrations (MICs) of chitosan vary widely—from 0.005% to 1.5% (w/v) for *Staphylococcus aureus* (Chang et al., 1989; Wang, 1992) or from 0.025% to 1.0% (w/v) for *Escherichia coli* (Gerasimenko et al., 2003; Uchida et al., 1989), and its antibacterial effects seem to be closely related to MW and degree of acetylation (Lee et al., 2002). However, the results published so far have shown contradictory conclusions: some authors reported that chitosan is more effective than COS in inhibiting growth of bacteria—e.g. water-insoluble chitosans exhibit higher antimicrobial effect over *E. coli* than COS (Jeon et al., 2001; Qin et al., 2006), or COS have weak or no antimicrobial activity (No et al., 2002; Qin et al., 2006); others claimed that an increase in MW leads to a decrease in the activity of chitosan—e.g. COS exhibit higher antimicrobial effect over *E. coli* than water-insoluble chitosans (Xia and Wu, 1996; Zheng and Zhu, 2003), or the high number of amino groups in chitosan molecules (for MW above 91.6 kDa) leads to a decrease in antimicrobial activity (Liu et al., 2001).

On the other hand, the antibacterial activity of chitosan has been demonstrated almost exclusively *in vitro* (either using liquid or solid media); however, such results can hardly be extrapolated to complex food systems, because interaction of chitosans with food ingredients will likely interfere with the efficacy of the former—besides other important changes, which are simultaneously brought about by sensory and textural properties of the food matrix itself. Rhoades and Roller (2000) concluded that chitosan has a potential for use as food preservative, yet the food matrix constituents appear to play important roles upon its antimicrobial capacity. Kiskó et al. (2005), in turn, suggested that addition of chitosan to fruit juice during processing may increase the risk of poisoning by *E. coli* O157:H7, via knocking out a portion of the prevailing native yeast population. Ausar et al. (2002) showed that the inhibiting effects of chitosan upon three representative milk fermentation bacteria were reversed when that biopolymer was incubated with milk prior to exposure to those bacteria. Devlieghere et al. (2004) claimed that the power of chitosan as food antimicrobial agent is limited to food products that have low levels of protein and sodium chloride (e.g. fruits and vegetables), whereas No et al. (2002) reported that the addition of chitosan to acidic foods enhances its effectiveness as natural preservative. Finally, Lee et al. (2004) concluded that a packaging material coated with a combination of nisin and chitosan improves significantly the microbial stability of milk and orange juice, at 3° and 10 °C.

In view of the above data, this research effort was aimed at further elucidating the relationship between MW of chitosans and their antimicrobial activity upon selected common food-borne spoilage and pathogen microorganisms, as well as the effects of interaction with food ingredients upon the activity in typical liquid food products. Our study used *S. aureus* and *E. coli* as model microorganisms (one Gram-positive and one Gram-negative, respectively), and milk and apple juice as examples of food matrices (one rich in proteins and one rich in carbohydrates, respectively)—including, for the first time, sensory tests.

Materials and methods

Sources of chitosans, microorganisms and foods

Chitosans, characterized by three different MW (average of 628, 591 and 107 kDa), and with a degree of deacetylation in the range 80–85%, were obtained from Sigma-Aldrich (Sintra, Portugal).

Chitooligosaccharide mixtures, characterized by two distinct MW (designated as <5 and <3 kDa), and with a degree of deacetylation in the range 80–85%, were purchased from Nicechem (Shanghai, China). The chitosans and COS tested were obtained from crab shells.

Two food matrices were tested—UHT semi-skimmed milk containing 1.6% (w/v) milkfat, purchased from AGROS (Porto, Portugal); and pasteurized apple juice, obtained from Compal (Almeirim, Portugal).

Microorganisms were from NCTC (London, UK), viz. *E. coli* (NCTC 9001) and *S. aureus* (NCTC 8532).

Preparation of chitosans and COS solutions

In the preparation of chitosan solutions, 2.5% (w/v) chitosans were dispersed in a 1.0% (v/v) acetic acid solution, whereas COS solutions were prepared by dissolving 2.5% (w/v) COS in deionized water. In both cases, the pH was adjusted to 5.8 with 10 M NaOH (the pH most adequate to solubilize chitosan and without any antibacterial effect). After stirring overnight, the solutions were autoclaved at 120 °C for 15 min (thermostability under these conditions had been previously checked).

Determination of minimum, inhibitory and lethal concentrations

MICs were determined as the lowest concentrations of chitosan or COS at which microorganisms cannot grow in Muller–Hinton (M–H) broth (Lab M, UK); based on the method of Ruparelia et al. (2008), the strains were inoculated into M–H broth and incubated to the logarithmic growth phase at 37 °C. The growth density was adjusted to match a MacFarland 0.5 standard (10^8 CFU/ml). A 1:100 dilution was prepared in a fresh M–H broth, and used as the inoculum (10^6 CFU/ml); several concentrations were tested, and microbial growth was monitored via turbidity by 24 h of incubation at 37 °C.

Minimum lethal concentrations (MLCs) were determined as the lowest concentrations of chitosan or COS at which microbial growth was prevented, and the initial viability was in addition reduced by at least 99.9% within 24 h; it was determined by inoculation of $100\,\mu L$-aliquots of negative tubes (absence of turbidity in the MIC determination) on M–H agar, using the spread plate technique.

Time-death determinations invitro

The antimicrobial activities of the three (high, medium and low MW) chitosans, and of the two (<5 and <3 kDa) COS were tested as described previously by Borbone et al. (2006) with minor modifications, against *S. aureus* and *E. coli* in M–H broth, at three different inoculum levels -10^7, 10^5 and 10^3 CFU/mL. Both strains were adjusted to 10^8 CFU/mL in saline solution (turbidity equivalent to that of a 0.5 McFarland standard), and were further diluted at 1:10 to obtain the additional inoculum levels desired in M–H broth containing the following chitosan/COS concentrations: 0.50%, 0.25% and 0.10% (w/v). Viability counts of chitosan/COS-containing suspensions were performed at 0, 30, 60, 120 and 240 min, by the spread plate technique on plate count agar (PCA, from Lab M), for 24 h at 37 °C. Death curves were constructed by plotting log (N/N_0)—where N is the viable cell number at a given time and N_0 is the viable cell number at time zero, versus time. Bactericidal activity was defined as a >3 log cycle decrease of the initial inoculum size.

The antimicrobial activities of the three chitosans and the two COS were tested against *E. coli* and *S. aureus* in M–H broth (used as control), as well as in two selected (liquid) food matrices—milk and apple juice. Both strains were adjusted to 10^8 CFU/mL in saline solution (turbidity equivalent to that of a 0.5 McFarland standard), and were further diluted at 1:10 to obtain 10^5 CFU/mL in M–H broth (milk or apple juice) containing 0.50% (w/v) of chitosan/COS. Viability counts of chitosan/COS-containing suspensions were performed at 0, 1, 2, 4, 8, 24, 48, 72, 96 and 120 h, by the spread plate technique on PCA, for 24 h at 37 °C. Death curves were constructed by plotting $\log_{10}$ (CFU/mL) versus time. Controls to assess contamination in juice or milk were done in all sampling times, by performing the enumeration on PCA plates in order to assess if any contaminant or resident microorganism was present.

Determination of sensory profile

A panel of 12 trained subjects performed a sensory analysis. The said subjects were selected for their sensory ability (in order to achieve this purpose, three previous sensory tests were done, viz. off-flavor and basic taste identification, as well as triangular tests), and trained for descriptive analysis according to the standard sensory profile guidelines set by ISO 6564 (2003) (to become familiar with the sensory vocabulary).

Sensory changes (perceived by aspect, flavor and taste, as well as texture) of the apple juice caused by the addition of 0.50% (w/v) chitosan or COS were assessed via difference-from-control test—in which apple juice was used as control. Previously, studies with chitosan or COS added to milk samples were performed, but unsatisfactory results were obtained by panel sensory evaluation, in terms of visual and textural appearance. Each assessor smelled and tasted the samples, and quantified them using a −3 to +3 continuous scale: −3 stand for the weakest and +3 for the strongest.

Statistical analysis

Analyses were performed in triplicate, and each experiment was carried out in duplicate. Mean values and standard deviations were accordingly calculated from the experimental data obtained, and analysis of variance (ANOVA) was applied at a 5% level of significance, using chitosan/COS concentration, chitosan type and inoculum level as main factors. Pairwise comparisons were done using Bonferroni's test, at the same level of significance.

Results and discussion

Minimum inhibitory and lethal concentrations

The antimicrobial activities of chitosans and COS are depicted in Fig. 1. It can be easily observed that MICs and MLCs depend on the bacterium being studied, and on the MW of the active compound tested. Growth of *E. coli* was markedly inhibited by COS (Fig. 1a)—and the associated MICs were below 0.10% (w/v); this threshold is lower than that reported elsewhere (Gerasimenko et al., 2003)—1.0% (w/v) for a 5 kDa oligomer effect on growth, but slightly higher than that associated with a 6 kDa oligomer—0.06% (w/v). Furthermore, the inhibitory effect decreased slightly as MW increased—0.25% (w/v) of high MW chitosan was required to inhibit growth of *E. coli*. In the case of *S. aureus* (Fig. 1b), chitosans showed a stronger antibacterial activity than COS; apparently, chitosans exhibit a stronger bactericidal effect upon Gram-positive than Gram-negative bacteria (No et al., 2002). The MICs varied from 0.10% (in the case of high and medium MW chitosan) to 0.23% (w/v) (in the case of <3 kDa COS); these values are relatively high when compared with those obtained by Jeon et al. (2001)—0.06% and 0.12% (w/v) for chitosan and COS, respectively. In addition, the differences found between MICs and MLCs for *S. aureus* were more substantial than those obtained for *E. coli*: MLCs of COS for *S. aureus*, ca. 0.5% (w/v), were indeed more than 3-fold those of high and medium MW chitosans, ca. 0.15% (w/v).

Differences in MIC (or MLC) values are recurrent in studies encompassing chitosan, especially when various MWs are tested in attempts to find the one that exerts the highest antibacterial effect. It has been claimed (Jeon et al., 2001) that 10 kDa is the minimum MW required for inhibition; however, Zheng and Zhu (2003) showed that a mixture of 0.25% (w/v) COS with MW <5 kDa yielded the highest inhibition over *E. coli*, whereas a 305 kDa fraction had the highest effect against *S. aureus,* at a similar concentration. Antibacterial activity over *E. coli* brought about by oligomers (i.e. trimers to hexamers) was also reported (Jeon and Kim, 2000)—even at 0.01% (w/v), while Ueno et al. (1997) claimed that 2.2 kDa oligomers caused little effect on microbial growth. Furthermore, the optimum MW found by Xia et al. (1996) was 1.5 kDa; and No et al. (2002) reported that the most effective MW against those two bacteria is 470 kDa, when the testing range was 1–1671 kDa; in both cases, MICs were ca.
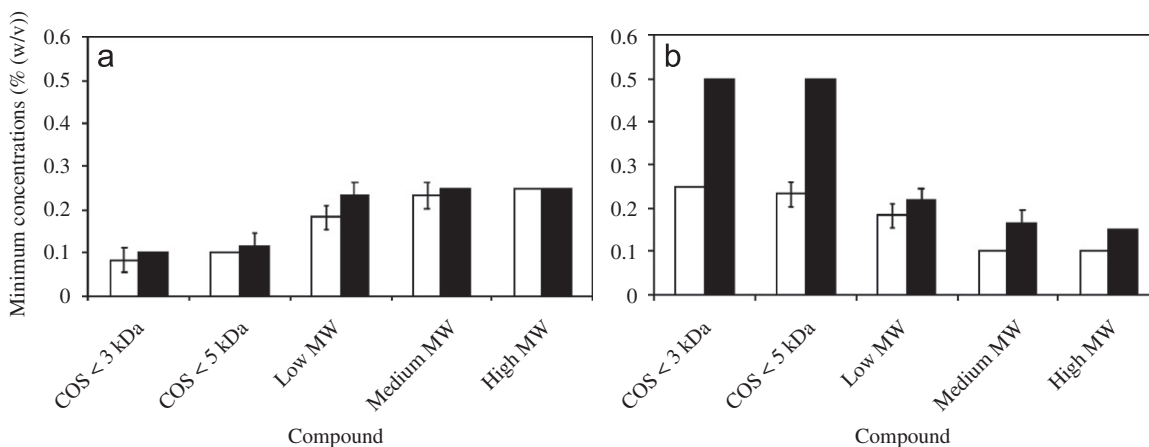

Fig. 1. MIC (□) and MLC (■) of chitosans of various MWs and chitooligosaccharides, COS, upon (a) *E. coli* and (b) *S. aureus* (average ± standard deviation).

0.08% (w/v). The aforementioned differences are probably accounted for by the distinct experimental conditions used by different authors—viz. the MW range, the degree of deacetylation, the concentration, the final pH, the bacteria and chitosan sources, and the solvents employed, among other variables (Wang, 1992).

Antibacterial activity in vitro

The effects of chitosan or COS at different concentrations upon 10^7 and 10^5 CFU/mL inocula of E. coli are depicted in Fig. 2. At the lowest inoculum tested, i.e. 10^3 CFU/mL (results not shown), all compounds revealed bactericidal activity, with viable numbers reduced by up to 3 log cycles within 4 h of exposure. In this case, the most relevant features were the differences in time required to achieve said reduction versus chitosan concentration and MW: e.g. the < 3 kDa COS mixture needed 30 min to achieve 99.9% reduction at 0.50% (w/v), whereas 120 min had to elapse for an identical reduction in the case of 0.10% (w/v). The three chitosans led, nevertheless, to similar results for the three concentrations: it took 60, 120 and 240 min for 0.1%, 0.25% and 0.50% (w/v) solutions to attain a 3 log cycle reduction. These results are in agreement with data published previously (Jeon and Kim, 2000; Liu et al., 2001): the antibacterial effect upon E. coli was found to increase with chitosan concentration, as in principle would be expected.

Regarding the higher inocula (i.e. 10^7 and 10^5 CFU/mL), our results revealed similar tendencies; when a 10^7 CFU/mL inoculum was used, only the COS mixtures showed bactericidal effect

(i.e. a reduction by 99.9% of the initial viable numbers), at 0.5% and 0.25% (w/v) (results not shown), whereas in general the three chitosans led only to a minor reduction of the initial population—not above 3 log cycles by 4 h. At the lowest concentration, 0.10% (w/v), only the < 3 kDa COS mixture showed bactericidal effect, although such an effect was rather similar to that of its < 5 kDa COS counterpart. The medium and high MW chitosans exhibited only a 2 log cycle reduction, but their low MW counterpart showed a slightly stronger effect. At 10^5 CFU/mL inoculum level, a solution of 0.50% (w/v) COS/chitosans (Fig. 2a) produced a reduction above 3 log cycles, although it was attained within different time periods—ca. 30 min for COS, and more than 2 h for chitosans. By 2 h, the COS mixtures had essentially eliminated the initial bacterial population; a 5 log cycle-reduction in viable numbers was also achieved with 0.25% (w/v) COS mixtures, but only by 4 h (Fig. 2b)—these being the only two compounds that produced a reduction above 99.9%. At this inoculum level, both types of COS at 0.10% (w/v) led to a lower reduction (3 log cycles) by 4 h (Fig. 2c). The statistical analyses applied to the whole dataset indicated that all three factors (i.e. chitosan type, concentration and inoculum level) were statistically significant ($p < 0.05$); statistical significance also held for pairwise comparisons in terms of chitosan concentration and inoculum level, although no significant differences were found between COS (< 3 and < 5 kDa) and between chitosans (low, medium and high MW).

The results obtained with S. aureus were, in general, different—and, in some cases, even opposite to those encompassing
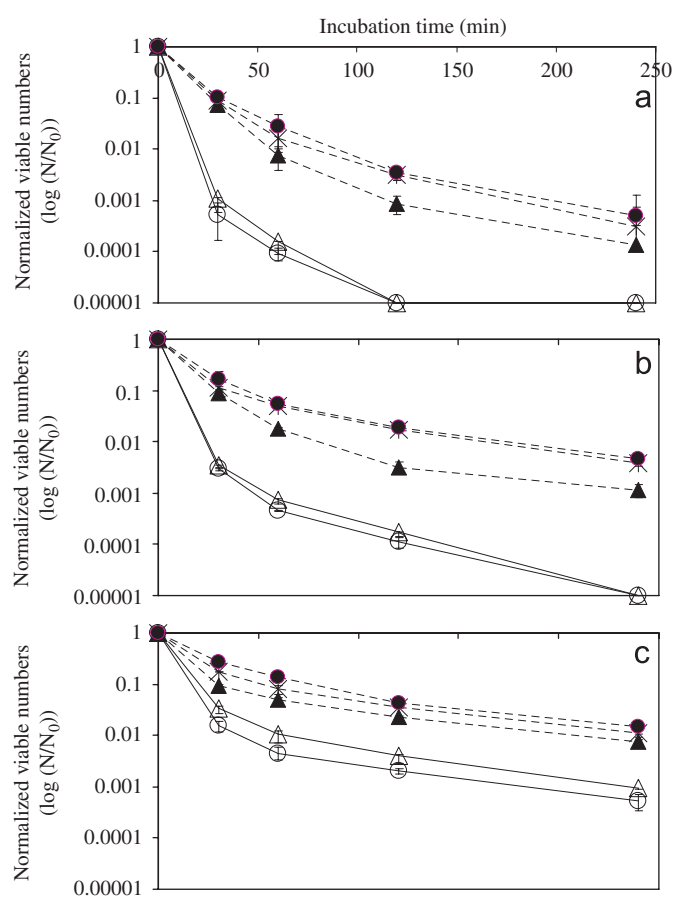
Fig. 2. Survival of E. coli, inoculated at 10^5 CFU/mL in M–H broth, in the presence of COS with < 3 (—○—) and < 5 kDa (—△—), and chitosans with low MW (···▲···), medium MW (—✳—) and high MW (···●···), at (a) 5.0 mg/mL, (b) 2.5 mg/mL and 1.0 mg/mL (c), throughout incubation time (average ± standard deviation).
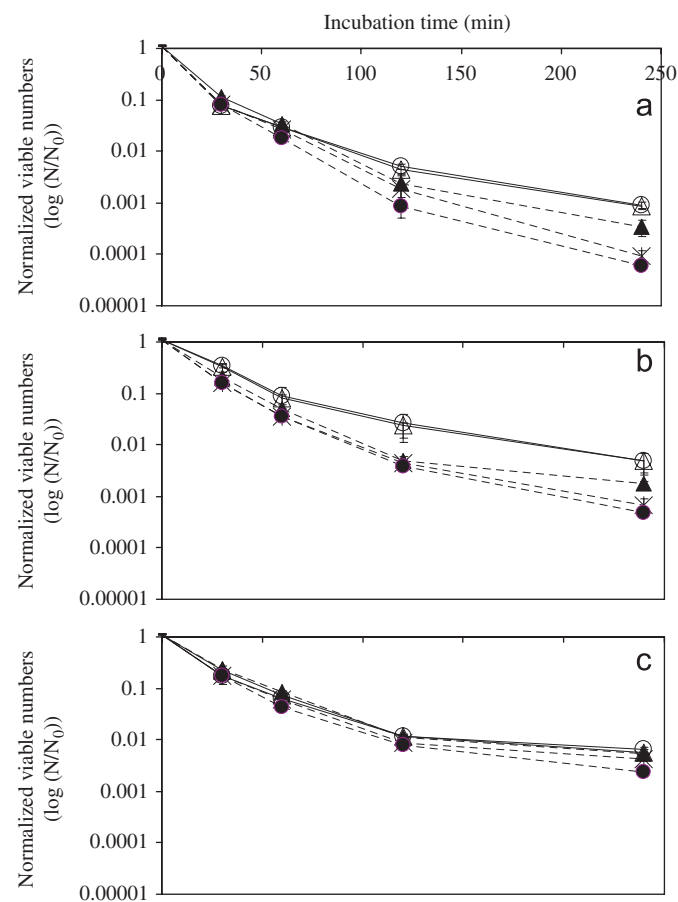
Fig. 3. Survival of S. aureus, inoculated at 10^5 CFU/mL in M–H, in the presence of COS with < 3 (—○—) and < 5 kDa (—△—), and chitosans with low MW (···▲···), medium MW (—✳—) and high MW (···●···), at (a) 5.0 mg/mL, (b) 2.5 mg/mL and 1.0 mg/mL (c), throughout incubation time (average ± standard deviation).

E. coli, especially concerning the effect of MW (Fig. 3). At the lowest inoculum -10^3 CFU/mL (results not shown), all five compounds tested exhibited a bactericidal effect against *S. aureus.* At 0.50% (w/v), all those compounds required only 30 min to completely eliminate the initial population of *S. aureus*, whereas high and medium MW chitosans at 0.25% (w/v) exerted their activity earlier (by 30 min) than the other three compounds (by 60 min). At the lowest concentration tested, high MW chitosan was more efficient—as it required only 1 h, instead of 4 h as found for the other compounds, to essentially eliminate the initial population. Analyzing the other two inocula (10^7 and 10^5 CFU/mL), the influence of MW was not as pronounced as for *E. coli.* For both inocula and for each of the three concentrations, a difference above 1 log cycle was never observed throughout 4 h for any of the five different compounds. At 10^7 CFU/mL, the high MW chitosan showed a stronger effect at all three concentrations tested (results not shown), followed by the medium MW chitosan; these two compounds were the only ones that presented a reduction higher than 99.9% at 0.5% (w/v). For the three concentrations considered and for both inoculum levels (10^7 and 10^5 CFU/mL), COS displayed the lowest antibacterial activity (Fig. 3). All chitosans exposed to the 10^5 CFU/mL inoculum caused a reduction above 99.9% at 0.5% (w/v) (Fig. 3a). In the case of high and medium MW chitosans, the 0.25% (w/v) concentration also showed bactericidal effect (Fig. 3b)—which could not be observed for any of the compounds tested at the lowest concentration, 0.10% (w/v) (Fig. 3c). The analyses encompassing *S. aureus* indicated that all factors (concentration, MW and inoculum level) were statistically significant ($p < 0.05$). However, pairwise comparisons revealed significant differences only between the extreme chitosan concentrations (i.e. 0.10% and 0.50%, w/v). The results encompassing both *S. aureus* and *E. coli* showed a clear tendency concerning influence of polymer chain length upon antibacterial action: the antimicrobial effect was strongly dependent on the type of target microorganism (Gram-negative versus Gram-positive) and the MW of chitosan. Antimicrobial activity was observed for the polymer with lower MW in the case of the Gram-negative bacterium, and the reverse in the case of the Gram-positive one. This conclusion is consistent with findings by other authors (No et al., 2002; Xia and Wu, 1996); it was furthermore suggested

(Zheng and Zhu, 2003) that the apparent differential action upon Gram-positive and Gram-negative microorganisms probably results from the intrinsic difference in their cell wall structure: it is easier for oligomers to penetrate the Gram-negative cell wall, whereas a mechanical barrier is formed by higher MW chitosans in their Gram-positive counterparts, which prevents nutrient absorption. Using confocal laser scanning microscopy, it has been shown (Liu et al., 2001) that COS actually penetrate *E. coli* cells, hence suggesting that its antibacterial activity is chiefly caused by inhibition of DNA transcription.

Antibacterial activity in food

The effect of MW upon the activity of chitosan against *S. aureus,* in culture medium (used as control), is shown in Fig. 4a. At the concentration selected -0.5% (w/v), all five compounds showed bactericidal activity. Although every chitosan or COS led to complete elimination of the initial population by 24 h, chitosans exhibited higher activity than COS at initial stages—a difference of almost 2 log cycles was actually observed by 8 h. As described above for the 4 h experiment, the COS mixtures exhibited similar antimicrobial effect at that concentration, whereas higher MW compounds showed a stronger activity. Strong bactericidal activity was also observed towards *E. coli,* but at different rates—COS needed only 2 h, whereas chitosans required an extra 6 h to deplete the initial viable cells (Fig. 4b).

Concerning milk, it was possible to notice that after an initial positive inhibitory effect that lasted for 8 h, COS mixtures allowed the viable counts of *S. aureus* to increase afterwards (Fig. 4c) until they reached values similar to that of the control. This result is likely explained by the high reactivity of those molecules with milk proteins (e.g. casein), which are anionic molecules (Chakraborty and Basak, 2007)—thus leading to loss of efficacy, since a portion of the COS molecules may be trapped in the protein network. This loss of efficiency has been reported elsewhere (Ausar et al., 2002) with milk fermentative bacteria: casein and milk fat, possibly due to a strong interaction with these polymers, could act in a competitive manner—and hence affect the antimicrobial activity of chitosan, and especially COS. Devlieghere et al. (2004) also
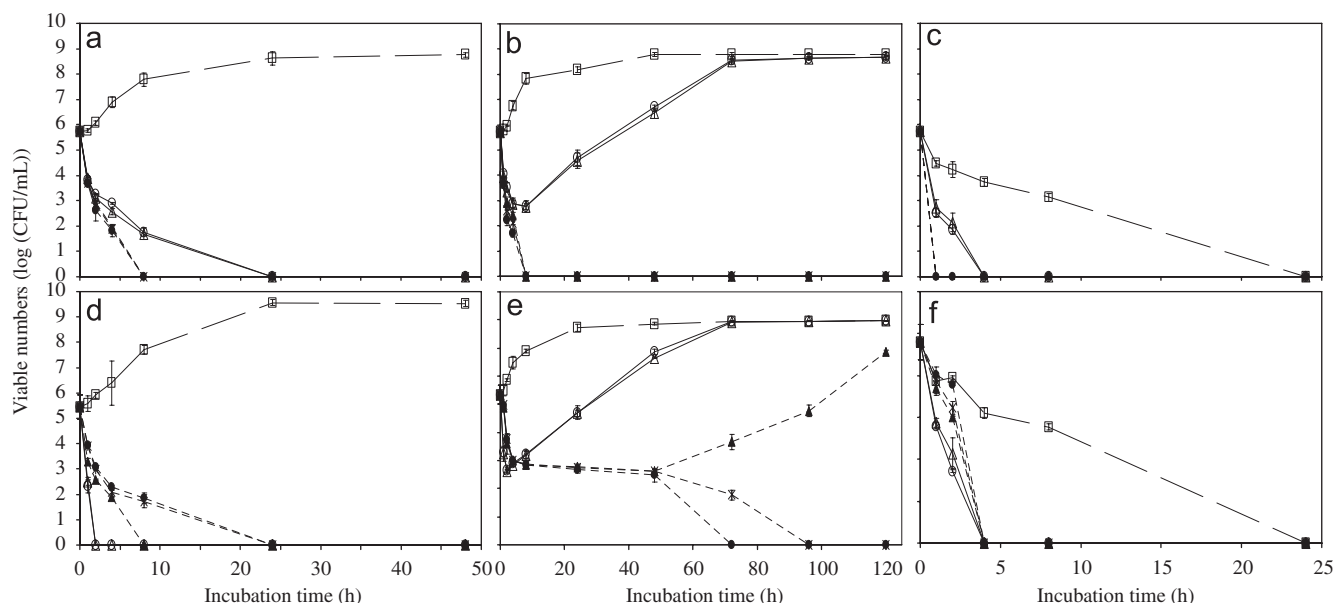
Fig. 4. Survival of (a) *S. aureus* and (b) *E. coli* in M–H broth, (c) *S. aureus* and (d) *E. coli* in semi-skimmed milk, and (e) *S. aureus* and (f) *E. coli* in apple juice, in the presence of COS with <3 (—○—) and <5 kDa (—△—), and chitosans with low MW (⋯▲⋯), medium MW (—✳—) and high MW (⋯●⋯), at 5 mg/mL, throughout incubation time (average ± standard deviation). (Control, —□—).

suggested that such a phenomenon could result from competition between negative charges on the milk protein and on the cell surface of the microorganism, for the positive charges on chitosan. However, for the three chitosans tested, no important differences were observed, as the highest MW apparently prevented loss of their antibacterial effect. In the case of *E. coli* (Fig. 4d), similar results were obtained for COS; this confirmed their strong interaction with the constituents of the milk matrix. By 48 h, only medium and high MW chitosans exhibited bactericidal activity, whereas low MW chitosan behavior resembled that of COS. In this case, pH did not influence the antibacterial activity, as its final value was essentially similar in all cases—e.g. for *E. coli* and by 120 h, the pH values of the solution with MW <3 kDa COS, the control and the high MW chitosan were 5.19, 5.32 and 5.41, respectively.

The results obtained with apple juice showed some degree of antimicrobial effect, even without addition of chitosans or COS: the starting bacterial population vanished by 24 h, most probably due to the low pH prevailing in that food (ca. 3.85). When chitosans or COS were added in the presence of *S. aureus* (Fig. 4e), there was a high death rate in all cases—which was similar among chitosans and COS: by 1 h, initial viable counts were reduced to< 10 CFU/mL by chitosans, whereas the same held for COS by 4 h. When in the presence of *E. coli* (Fig. 4f), the aforementioned compounds required 4 h to cause depletion (i.e. a 5 log cycle reduction) of the bacteria. Accordingly, such a fast action of chitosans and hydrolysates there of may be explained by the lower pH of this matrix—in agreement with claims by other authors (Roller and Covill, 1999; Tsai and Su, 1999; Wang, 1992) that the antimicrobial activity of chitosan is affected by pH, with a higher activity being recorded at a lower pH. For both microorganisms tested, the effect of the food matrix was found to be statistically significant ($p < 0.05$), as well as the MW and the incubation time. However, pairwise comparisons revealed no statistically significant differences between COS (<3 and <5 kDa), and between medium and high MW chitosans.

Sensory profile

When milk was used as model food, relatively large clusters formed by 2–4 h in presence of COS mixtures, when exposed to either microorganism. Conversely, no clusters were formed for high and medium MW chitosans, rather a net-like structure developed all over the food matrix. The five compounds produced changes that made the semi-skimmed milk unsuitable for consumption—from visual and textural points of view, so no further sensory testing was warranted. Concerning apple juice, such unfavorable changes were not perceived, so it was formally submitted to evaluation by a trained panel. The results (Table 1) showed a general trend: an increase in the magnitude of changes with an increase in MW. Such undesirable characteristics as astringency, after taste or high viscosity were more noticeable when chitosans were added—especially high MW ones, than when COS mixtures were considered. In addition, characteristics desirable for apple juice, e.g. taste or aroma of fresh apples, tended to vanish as the MW increased. Furthermore, COS maintained several typical characteristics of traditional apple juice—viz sweetness and acidity, or resembled even more the apple feedstock—with references to boiled apple/apple jam keynotes.

Conclusions

The antimicrobial effect of chitosans is strongly dependent on the type of target microorganism (Gram-negative versus Gram-positive) and the MW of chitosan—being higher for lower MW in the case of the Gram-negative bacterium, and the reverse in the case of the Gram-positive one. The stronger antibacterial activity observed, at lower pH, in the juice matrix, coupled with the poor performance of the milk matrix, suggest that the use of chitosans (irrespective of MW) will be limited to food products that possess a low protein content. However, the interference of proteins upon chitosan/COS inhibiting effects decreases with increase of MW. Chitosans and oligomers thereof are also more effective as food preservatives in low pH foods. Finally, COS appear in general as more promising for the final consumer.

Acknowledgments

Table 1
Sensory differences assessed by a group of trained panellists, between apple juice containing 0.50% (w/v) of chitosan/COS, and plain apple juice (control), expressed in a scale ranging from −3 to 3 : −3 = weaker than, 0 = equal to, and 3 = stronger than (average ± standard deviation)

	Control	High MW	Low MW	COS <3 kDa
Aspect				
Color	−0.1 ± 0.2	1.1 ± 0.1	1.3 ± 0.1	2.1 ± 0.1
Translucency	0.0 ± 0.1	0.2 ± 0.2	0.1 ± 0.2	0.0 ± 0.2
Aroma				
Global intensity	0.0 ± 0.0	0.9 ± 0.3	0.6 ± 0.2	0.5 ± 0.2
Fresh apple	0.2 ± 0.4	−0.7 ± 0.3	−0.4 ± 0.3	−0.1 ± 0.2
Boiled apple/apple jam	0.1 ± 0.4	0.2 ± 0.3	0.3 ± 0.3	0.7 ± 0.2
Others (seafood)	0.0 ± 0.0	1.5 ± 0.2	−0.2 ± 0.2	−0.3 ± 0.1
Taste				
Sweetness	0.2 ± 0.1	−0.3 ± 0.3	0.1 ± 0.5	0.4 ± 0.3
Acidity	0.2 ± 0.1	1.0 ± 0.2	1.2 ± 0.2	0.2 ± 0.1
Intensity	0.2 ± 0.1	0.3 ± 0.5	0.8 ± 0.4	0.3 ± 0.3
Fresh apple	0.1 ± 0.1	−1.6 ± 0.3	−1.2 ± 0.2	−0.2 ± 0.2
Boiled apple/apple jam	−0.1 ± 0.3	−0.9 ± 0.5	−0.4 ± 0.4	0.5 ± 0.3
Richness	0.1 ± 0.1	0.1 ± 0.4	0.2 ± 0.5	0.1 ± 0.2
Persistence	0.3 ± 0.1	1.1 ± 0.4	0.2 ± 0.6	0.6 ± 0.2
After taste	0.3 ± 0.1	1.7 ± 0.4	1.5 ± 0.5	0.9 ± 0.3
Others (bitter)	0.0 ± 0.0	2.4 ± 0.2	2.1 ± 0.0	0.0 ± 0.0
Texture				
Texture in mouth/viscosity	0.0 ± 0.0	1.4 ± 0.3	1.6 ± 0.3	0.7 ± 0.4
Astringency	0.2 ± 0.1	2.3 ± 0.1	2.4 ± 0.2	1.3 ± 0.3

References

Ausar, S.F., Passalacqua, N., Castagna, L.F., Bianco, I.D., Beltramo, D.M., 2002. Growth of milk fermentative bacteria in the presence of chitosan for potential use in cheese making. Int. Dairy J. 12, 899–906.

Borbone, S., Cascone, C., Santagati, M., Mezzatesta, M.L., Stefani, S., 2006. Bactericidal activity of ertapenem against major intra-abdominal pathogens. Int. J. Antimicrob. Agents 28, 396–401.

Chae, S.Y., Jang, M.K., Nah, J.W., 2005. Influence of molecular weight on oral absorption of water soluble chitosans. J. Controlled Release 102, 383–394.

Chakraborty, A., Basak, S., 2007. pH-Induced structural transitions of caseins. J. Photochem. Photobiol., B: Biol. 87, 191–199.

Chang, D.S., Cho, H.R., Goo, H.Y., Choe, W.K., 1989. A development of food preservative with the waste of crab processing. Bull. Korean Fish Soc. 22, 70–78.

Chien, P.J., Sheu, F., Huang, W.T., Su, M.S., 2007. Effect of molecular weight of chitosans on their antioxidative activities in apple juice. Food Chem. 102, 1192–1198.

Devlieghere, F., Vermeiren, L., Debevere, J., 2004. New preservation technologies: possibilities and limitations. Int. Dairy J. 14, 273–285.

Gerasimenko, D.V., Avdienko, I.D., Bannikova, G.E., Zueva, O.Y., Varlamov, V.P., 2003. Antibacterial effects of water-soluble low molecular-weight chitosans on different microorganisms. Appl. Biochem. Microbiol. 40, 253–257.

Giustina, A., Ventura, P., 1995. Weight-reducing regimens in obese subjects: effects of a new dietary fibre integrator. Acta Toxicol. Ther. 16, 199–214.

ISO 6564, 2003. Analyse sensorielle-methodologie—Méthodes d'établissement du profil de la flaveur. Organisation Internationale de Normalisation, Geneva, Switzerland ISO 6564:2003.

Jeon, Y.J., Kim, S.K., 2000. Production of chitooligosaccharides using an ultrafiltration membrane reactor and their antibacterial activity. Carbohydr. Polym. 41, 133–141.

Jeon, Y.J., Park, P.J., Kim, S.K., 2001. Antimicrobial effect of chitooligosaccharides produced by bioreactor. Carbohydr. Polym. 44, 71–76.

Kim, S.K., Rajapakse, N., 2005. Enzymatic production and biological activities of chitosan oligosaccharides (COS): a review. Carbohydr. Polym. 62, 357–368.

Kim, D.G., Jeong, Y.I., Choi, C., Roh, S.H., Kang, S.K., Jang, M.K., Nah, J.W., 2006. Retinol-encapsulated low molecular water-soluble chitosan nanoparticles. Int. J. Pharm. 319, 130–138.

Kiskó, G., Sharp, R., Roller, S., 2005. Chitosan inactivates spoilage yeasts but enhances survival of Escherichia coli O157:H7 in apple juice. J. Appl. Microbiol. 98, 872–880.

Koide, S.S., 1998. Chitin–chitosan: properties, benefits and risks. Nutr. Res. 18, 1091–1101.

Lee, H.W., Park, Y.S., Jung, J.S., Shin, W.S., 2002. Chitosan oligosaccharides, dp 2-8, have prebiotic effect on the Bifidobacterium bifidium and Lactobacillus sp. Anaerobe 8, 319–324.

Lee, S.H., Suh, J.S., Kim, H.S., Lee, J.D., Song, J., Lee, S.K., 2003. MR evaluation of radiation synovectomy of the knee by means of intra-articular injection of holmium-166-chitosan complex in patients with rheumatoid arthritis: results at 4-month follow-up. Korean J. Radiol. 4, 170–178.

Lee, C.H., Park, H.J., Lee, D.S., 2004. Influence of antimicrobial packaging on kinetics of spoilage microbial growth in milk and orange juice. J. Food Eng. 65, 527–531.

Lim, S.H., Hudson, S.M., 2004. Application of a fiber-reactive chitosan derivative to cotton fabric as an antimicrobial textile finish. Carbohydr. Polym. 56, 227–234.

Liu, X.F., Guan, Y.L., Yang, D.Z., Li, Z., Yao, K., 2001. Antibacterial action of chitosan and carboxymethylated chitosan. J. Appl. Polym. Sci. 79, 1324–1335.

Liu, N., Chen, X.G., Park, H.J., Liu, C.G., Liu, C.S., Meng, X.H., Yu, L.J., 2006. Effect of MW and concentration of chitosan on antibacterial activity of Escherichia coli. Carbohydr. Polym. 64, 60–65.

Liu, B., Liu, W.S., Han, B.Q., Sun, Y.Y., 2007. Antidiabetic effects of chitooligosaccharides on pancreatic islet cells in streptozotocin-induced diabetic rats. World J. Gastroenterol. 13, 725–731.

No, H.K., Park, N.Y., Lee, S.H., Meyers, S.P., 2002. Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. Int. J. Food Microbiol. 74, 65–72.

Okuyama, K., Noguchi, K., Kanenari, M., Egawa, T., Osawa, K., Ogawa, K., 2000. Structural diversity of chitosan and its complexes. Carbohydr. Polym. 41, 237–247.

Ormrod, D.J., Holmes, C.C., Miller, T.E., 1998. Dietary chitosan inhibits hypercholesterolaemia and atherogenesis in the apolipoprotein E-deficient mouse model of atherosclerosis. Atherosclerosis 138, 329–334.

Pospieszny, H., Struszczyk, H., Chirkov, S.N., Atabekov, J.G., 1994. New applications of chitosan in agriculture. In: Karniki, S.Z. (Ed.), Chitin World. Wirtschaftsverlag, Bremerhaven, pp. 246–254.

Qin, C., Li, H., Xiao, Q., Liu, Y., Zhu, J., Du, Y., 2006. Water-solubility of chitosan and its antimicrobial activity. Carbohydr. Polym. 63, 367–374.

Ravi-Kumar, M.N., Muzzarelli, R.A., Muzzarelli, C., Sashiwa, H., Domb, A.J., 2004. Chitosan chemistry and pharmaceutical perspectives. Chem. Rev. 104, 6017–6084.

Rhoades, J., Roller, S., 2000. Antimicrobial actions of degraded and native chitosan against spoilage organisms in laboratory media and foods. Appl. Environ. Microbiol. 66, 80–86.

Rinaudo, M., 2006. Chitin and chitosan: properties and applications. Prog. Polym. Sci. 31, 603–632.

Roller, S., Covill, N., 1999. The antifungal properties of chitosan in laboratory media and apple juice. Int. J. Food Microbiol. 47, 66–77.

Ruparelia, J.P., Chatterjee, A.K., Duttagupta, S.P., Mukherji, S., 2008. Strain specificity in antimicrobial activity of silver and copper nanoparticles. Acta Biomater. 4, 707–716.

Seo, S., King, J.M., Prinyawiwatkul, W., 2007. Simultaneous depolymerization and decolorization of chitosan by ozone treatment. J. Food Sci. 72, 522–526.

Shahidi, F., Arachchi, J.K.V., Jeon, Y.J., 1999. Food applications of chitin and chitosans. Trends Food Sci. Technol. 10, 37–51.

Shi, C., Zhu, Y., Ran, X., Wang, M., Su, Y., Cheng, T., 2006. Therapeutic potential of chitosan and its derivatives in regenerative medicine. J. Surg. Res. 133, 185–192.

Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S., Suzuki, M., 1986. Antitumor effect of hexa-N-acetylchitohexaose and chitohexaose. Carbohydr. Res. 151, 403–408.

Tikhonov, V.E., Stepnova, E.A., Babak, V.G., Yamskov, I.A., Guerrero, J.P., Jansson, H.B., Llorca, L.V., Salinas, J., Gerasimenko, D.V., Avdienko, I.D., Varlamov, V.P., 2006. Bactericidal and antifungal activities of a low molecular weight chitosan and its N-/2(3)-(dodec-2-enyl)-succinoyl/-derivatives. Carbohydr. Polym. 64, 66–72.

Tsai, G.J., Su, W.H., 1999. Antibacterial activity of shrimp chitosan against Escherichia coli. J. Food Prot. 62, 239–243.

Uchida, Y., Izume, M., Ohtakara, A., 1989. Preparation of chitosan oligomers with purified chitosanase and its application. In: Skjåk-Brœk, G., Anthonsen, T., Sandford, P. (Eds.), Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications. Elsevier Applied Science, Barking, pp. 373–382.

Ueno, K., Yamaguchi, T., Sakairi, N., Nishi, N., Tokura, S., 1997. Antimicrobial activity by fractionated chitosan oligomers. In: Domard, A., Roberts, G.A.F., Varum, K.M. (Eds.), Advances in Chitin Science, vol. II. Jacques André, Lyon, pp. 156–161.

Vernazza, C.L., Gibson, G.R., Rastall, R.A., 2005. In vitro fermentation of chitosan derivatives by mixed cultures of human faecal bacteria. Carbohydr. Polym. 60, 539–545.

Wang, G.H., 1992. Inhibition and inactivation of five species of foodborne pathogens by chitosan. J. Food Prot. 55, 916–919.

Woodgate, D.E., Conquer, J.A., 2003. Effects of a stimulant-free dietary supplement on body weight and fat loss in obese adults: a six-week exploratory study. Curr. Ther. Res. 64, 248–262.

Xia, W.S., Wu, Y.N., 1996. Functional properties of chitooligosaccharides. J. Wuxi Univ. Light Ind. 15, 297–302.

Zheng, L.Y., Zhu, J.F., 2003. Study on antimicrobial activity of chitosan with different molecular weights. Carbohydr. Polym. 54, 527–530.