J. Microbiol. Biotechnol. (2010), **20**(2), 311–318
doi: 10.4014/jmb.0904.04038
First published online 14 November 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

In Vitro Screening for Antimicrobial Activity of Chitosans and Chitooligosaccharides, Aiming at Potential Uses in Functional Textiles

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Received: April 22, 2009 / Revised: August 4, 2009 / Accepted: August 21, 2009

Antimicrobial finishing of textiles has been found to be an economical way to prevent (or treat) skin disorders. Hence, this research effort was aimed at elucidating the relationship between the molecular weight (MW) of chitosan and its antimicrobial activity upon six dermal reference microorganisms, as well as the influence of the interactions with cotton fabrics on said activity. Using 3 chitosans with different MWs, as well as two chitooligosaccharide (COS) mixtures, a relevant antimicrobial effect was observed by 24 h for the six microorganisms tested; it was apparent that the antimicrobial effect is strongly dependent on the type of target microorganism and on the MW of chitosan – being higher for lower MW in the case of *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, and the reverse in the case of both Gram-positive bacteria. Furthermore, a strong antifungal effect was detectable upon *C. albicans*, resembling the action over Gram-positive bacteria. Interactions with cotton fabric resulted in a loss of COS activity when compared with cultured media, relative to the effect over Gram-negative bacteria. However, no significant differences for the efficacy of all the 5 compounds were observed by 4 h. The three chitosans possessed a higher antimicrobial activity when impregnated onto the fabric, and presented a similar effect on both Gram-positive bacteria and yeast, in either matrix. *Pseudomonas aeruginosa* showed to be the most resistant microorganism to all five compounds.

Keywords: Molecular weight, biopolymers, skin-borne microorganisms

Textile products, especially those obtained from natural fabrics, can provide a particularly suitable environment for microorganisms to grow owing to their large specific surface area and capacity to retain moisture. Hence, the use of antibacterial agents to prevent, or at least retard, growth of bacteria is becoming a standard finish for textile goods. A treatment that combines a nontoxic and biodegradable agent, with antimicrobial and anti-inflammatory actions, would thus be desirable to prevent the aforementioned situation, besides other natural skin conditions [17]. To this deed, such biological polymers as chitosan have recently attracted a great deal of attention, because of a number of desirable features.

Full (or partial) deacetylation of chitin produces chitosan, a linear polysaccharide composed mainly of β-1,4-2-deoxy-2-amino-D-glucopyranose, and β-1,4-2-deoxy-2-acetamido-D-glucopyranose residues to a lesser extent [37]. Evidence has been put forward that chitosan possesses various biological activities (*viz.*, antioxidant [1], wound-healing accelerator [21], and antibacterial and antifungal [19, 26, 32 35]). These features, combined with its biocompatibility and biodegradability [10], make it an interesting polymer for several applications in the textile industry [16], in the pharmaceutical industry, which includes wound dressings, gauzes, and medical sutures [40], in the cosmetic industry [13, 25], in the food industry [4], and also in medicine [3]. However, its high molecular weight (MW), which hampers solubility in acid-free aqueous media, has limited its practical applications.

Recent studies pertaining to chitosan have focused on the conversion thereof to oligosaccharides, because the latter are not only water-soluble [14], but also possess versatile functional properties; for example, antitumor [34] and immunostimulatory [29], in addition to antifungal and antimicrobial [35], and including in particular enhancement of protection against infection by a few pathogens [33]. The reported minimum inhibitory concentrations (MICs) of chitosan vary widely with the bacteria at stake, from 0.005% to 1.5% (w/v) [11, 22, 28], and its antibacterial effect seems to be closely related to MW [15]. Polymeric chitosan

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exhibits higher antibacterial activity against Gram-positive than -negative bacteria. On the contrary, chitooligosaccharides (COS) reveal better activity against Gram-negative bacteria [22, 41, 42]. The antibacterial activity, besides being influenced by the degree of polymerization, also depends on the level of deacetylation, type of target microorganism, and solvent, and is inversely affected by pH, with higher activity seen at low pH values [14, 23, 38]. 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 textile goods, because interaction of chitosans with the fabric components will likely interfere with their efficacy.

In view of the above data, this research effort aimed at further elucidating the relationship between MW of chitosans and their antimicrobial activity, upon six epidermal reference microorganisms (*viz.*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Candida albicans*) as well as the influence of the interaction with cotton fabrics upon said activity.

Materials and Methods

Chitosans and Cotton Fabric

Chitosans, characterized by three distinct MWs (average of 628, 591, and 107 kDa), and possessing a degree of deacetylation in the range 80–85%, were obtained from Sigma-Aldrich (Sintra, Portugal). Chitooligosaccharide mixtures, characterized by two distinct MWs (designated as <5 and <3 kDa), and possessing a degree of deacetylation also in the range 80–85%, were purchased from Nicechem (Shanghai, China). The chitosans and COS tested were obtained from crab shells.

Organic cotton fabric was kindly offered by Crispim Abreu & Cia., Lda. (Riba d'Ave, Portugal).

Microorganisms

Microorganisms were obtained from ATCC (Barcelona, Spain); namely, *Escherichia coli* ATCC 25922, *Klebsiella peumoniae*, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus epidermidis* ATCC 155, and *Candida albicans* ATCC 18804 (all isolated from skin microflora).

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 (the most adequate pH to solubilize chitosan without any antibacterial effect) with 10 M NaOH. 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

Minimum inhibitory concentrations (MICs) were determined as the lowest concentrations of chitosan or COS at which microorganisms cannot grow in Müller–Hinton (M-H) broth (Lab M, U.K.); based on the method of Ruparelia *et al.* [27], the strains were inoculated at 37°C into M-H broth, and incubated to the exponential growth phase. 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; they were determined by inoculation of 100-μl aliquots of negative tubes (absence of turbidity in the MIC determination) on M-H agar, using the spread plate technique.

Determination of Antimicrobial Activity of Chitosans in Culture Medium

The antimicrobial activities of the three (high, medium, and low MW) chitosans, and of the two (<3 and <5 kDa) COS were tested against the six aforementioned skin microorganisms in M-H broth, at a selected concentration of 0.50% (w/v) chitosans/COS (*i.e.*, that guaranteed inhibition of all microorganisms under study). After inoculation, the medium was incubated for 4 h at 37°C, and samples were taken at 1 and 4 h. Each sample was diluted and plated by the spread plate technique on Plate Count Agar (PCA, from Lab M), followed by incubation at 37°C; the viable cell numbers were determined by 24 h (except *C. albicans*, which was incubated at 30°C for 36 h). The extent of survival was determined as 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.

Determination of Antimicrobial Activity of Chitosans in Cotton Fabric

Assessment of the antimicrobial activity was carried out according to the standard procedure described in the AATCC test method 100-2004. Rounds of cotton fabric (*ca.* 4.8 cm in diameter) were impregnated with 0.50% (w/v) chitosan/COS solutions using the pad-dry-cure method, followed by sterilization by exposure to UV for 15 min. Then, the entire surface was accordingly inoculated with 1 ml of a bacterial/yeast suspension containing *ca.* 10^5 CFU/ml, and incubated afterwards at 37°C (except for *C. albicans*, which was incubated at 30°C). Standard plate counts were performed by 1 and 4 h of incubation, and the percent reduction (R) was determined as R=100 (B–A)/B, where A represents the number of bacteria recovered from the inoculated treated rounds incubated over the desire contact period, and B represents the number of bacteria recovered from the inoculated treated rounds immediately after inoculation.

In order to assess the eventual loss of COS/chitosan during drying and sterilization, 10 rounds of cotton fabric for each compound were weighted to assess the mass differences before and after submission to those processes, but statistically significant differences were not observed between those compounds.

Surface Evaluation by Scanning Electron Microscopy of the Cotton Fabric

Scanning electron microscopy (SEM) and digital imaging analysis were applied in the visual examination of the cotton fabric surfaces, with and without treatment. The typical values used in this study ranged from 50 to 500×, which means a low magnification value,

but which is suitable for observation of the overall surface structure. In order to image our samples by SEM, rounds of cotton fabric (*ca.* 4.8 cm in diameter) were duly prepared and impregnated with 0.50% (w/v) chitosan/COS solutions, following the pad-dry-cure method; sterilization was by exposure to UV for 15 min. The samples were finally observed by a SEM (JSM-5600, from JEOL, Tokyo, Japan), under vacuum.

Statistical Analyses

Analyses were performed in triplicate, and each experiment was carried out in duplicate. Mean values and standard deviations were thus calculated from the experimental data obtained, and analysis of variance (ANOVA) was applied at a 5% level of significance, using chitosan type and incubation period 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 Table 1. It can easily be observed that both MICs and MLCs depend largely on the type of bacterium and on the MW of the chitosans. Chitooligosaccharides markedly inhibited the growth of *E. coli* and *K. pneumoniae* (see Table 1), and their MICs were below 0.10% (w/v). Furthermore, the inhibitory effect decreased slightly as the MW increased, where 0.25% (w/v) of high MW chitosan was required to inhibit growth of said microorganisms. The same inhibitory effect was observed on *P. aeruginosa*; however, it required higher chitosan concentrations to achieve a similar activity, ranging from 0.20% for the COS mixture with MW <3 kDa, to 0.50% (w/v) for the 3 chitosans tested. The higher effect of COS over Gram-negative bacteria had already been suggested previously [22, 42].

In the case of *S. aureus* and *S. epidermidis*, chitosans showed a stronger antibacterial activity than COS. Apparently, chitosan exhibits a stronger bactericidal effect upon Gram-positive than -negative bacteria [20]. The MICs varied from 0.10% (for both bacteria, in the case of high and medium MW) to 0.25% (w/v) (for both COS), or 0.20% regarding *S. epidermidis*. Our results suggested higher MICs than those reported by Jeon *et al.* [12]: 0.06% (w/v) of 685 kDa chitosan was required to inhibit either *S. aureus* or *S. epidermidis* growth, as well as *ca.* 0.12% (w/v) of COS (*ca.* 5 kDa) to inhibit *S. aureus*, and 0.25% (w/v) thereof to inhibit *S. epidermidis*. This observation may be explained by the higher deacetylation degree of chitosans/COS used by those authors (89%), which implies a higher number of side amine groups available for reaction; note that this rationale has been proposed by several authors as responsible for chitosans antibacterial action [23, 38, 39].

Encompassing *C. albicans*, the behavior was rather similar to the one detected in Gram-positive bacteria: the inhibitory effect increased slightly as the MW increased, where 0.25% (w/v) of each COS was needed to attain the same inhibitory effect as that caused by 0.15% (w/v) of high MW chitosan. A similar tendency for *C. albicans* was also suggested elsewhere [24], where COS (*ca.* 1.4–2.8 kDa) were reported to have no significant antimicrobial activity, whereas water-insoluble chitosans (*ca.* 400–48 kDa) exhibited an inhibitory effect against *C. albicans*.

Pertaining to MLCs, the tendency was essentially similar. The MLCs for *E. coli* and *K. pneumoniae* were rather similar to the MICs, whereas in the case of *P. aeruginosa* the concentrations required to attain the same bactericidal effect were 2-fold the MICs for COS mixtures, and above 0.50% (w/v) for the 3 chitosans. These results upon *P. aeruginosa* and *K. pneumoniae* are not consistent with those published elsewhere [6]: these authors claimed that 5 kDa COS could not suppress growth of either bacterium, even at 1.0% (w/v); however, the methodology used thereby (including higher pH values and distinct buffer), besides the bacterium source, was distinct. Regarding *S. aureus* and *C. albicans*, the MLCs observed for COS were 2-fold the MICs; conversely, no significant differences were found for *S. epidermidis*.

Numerous studies on the antibacterial activity of chitosan and its oligomers have been carried out, but controversial evidences on a correlation between antibacterial activity

Table 1. Minimum inhibitory and minimum lethal concentrations of different MW chitosans, against six skin-borne microorganisms.

Microorganisms	Molecular weight									
	High (628 kDa)		Medium (591 kDa)		Low (107 kDa)		COS (<5 kDa)		COS (<3 kDa)	
	MIC (%)	MLC (%)	MIC (%)	MLC (%)	MIC (%)	MLC (%)	MIC (%)	MLC (%)	MIC (%)	MLC (%)
E. coli	0.25	0.25	0.25	0.25	0.20	0.25	0.10	0.15	0.10	0.10
K. pneumoniae	0.25	0.25	0.25	0.25	0.20	0.25	0.10	0.15	0.10	0.15
P. aeruginosa	0.50	> 0.50	0.50	> 0.50	0.50	> 0.50	0.25	0.50	0.20	0.50
S. aureus	0.10	0.15	0.10	0.20	0.15	0.20	0.25	0.50	0.25	0.50
S. epidermidis	0.10	0.10	0.10	0.10	0.15	0.15	0.20	0.20	0.20	0.20
C. albicans	0.15	0.15	0.15	0.20	0.20	0.25	0.25	0.50	0.25	0.50

and chitosan MW have been reported. It was claimed [12] that 10 kDa is the minimum MW required for microorganism inhibition; this difference may be explained by the higher pH (6.00) used by the authors, which appears to negatively affect the antimicrobial effect, where an increase in pH leads to a decrease of the antibacterial action, since fewer amino groups in chitosan molecules will be free, as they become ionized at pH below 6 [2, 18]. However, Zheng and Zhu [42] (using similar conditions to those described in this paper; *e.g.*, pH,) reported that a mixture of 0.25% (w/v) chitooligosaccharides with MW <5 kDa yielded the highest inhibition over *E. coli*, whereas a 305-kDa fraction exhibited 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, even at 0.01% (w/v) [11], whereas 2.2-kDa oligomers were claimed to cause little effect on microbial growth [36]. Furthermore, an optimum MW of 1.5 kDa was found [41]; and the most effective MW against *S. aureus* and *E. coli* was reported to be 470 kDa, when the testing range was 1–1,671 kDa, knowing that, in both cases, the MICs were *ca.* 0.08% (w/v) [22]. The aforementioned differences are probably accounted for by the distinct experimental conditions used by those authors, namely, viz. the MW range, the degree of deacetylation (as already mentioned), the concentration, the final pH (which influences ionization of chitosan), the solvents employed (*e.g.*, acetate buffer, acetic acid, water, and lactic or formic acid), the sources of chitosan, and the origin of the bacteria as aforementioned [39].

Antimicrobial Activity of Chitosans/COS

The antimicrobial activity of chitosans/COS over the microorganisms tested in culture medium was already visible by 1 h of incubation. *Klebsiella pneumoniae* and

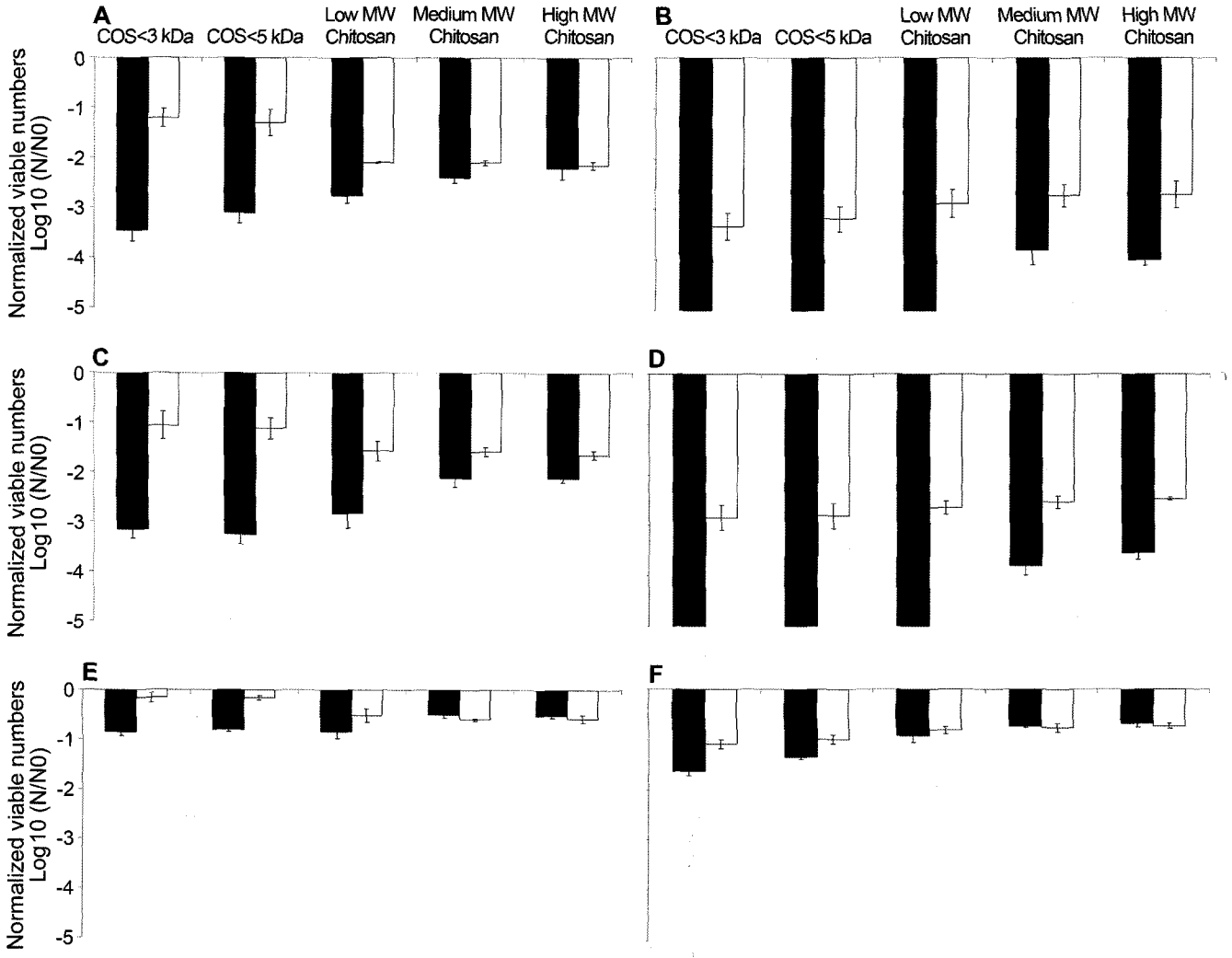

Fig. 1. Effects (average±standard deviation) of different MW chitosans and COS upon three Gram-negative bacteria [*E. coli* after 1 (**A**) and 4 h (**B**), *K. pneumoniae* after 1 (**C**) and 4 h (**D**), and *P. aeruginosa* after 1 (**E**) and 4 h (**F**)], incubated in Müller–Hinton broth (■) or cotton fabric (□).

E. coli presented similar trends, where a higher antibacterial activity of COS than chitosans was apparent. In fact, COS presented a bactericidal effect upon both bacteria (*i.e.*, a reduction above 3 log cycles by that time), whereas medium and high MW chitosans presented a lower reduction (*ca.* 2 log cycles). Encompassing *P. aeruginosa*, the resulting antibacterial activity was almost nil at this time (Fig. 1C). However, a trend similar to that described before for *E. coli* and *K. pneumoniae* was observed: the lower the MW, the (slightly) higher the antibacterial activity. This stronger effect of COS over those three bacteria, noticeable only after 1 h, is in agreement with the MICs found. This first hour was apparently not enough to differentiate the effects of the five compounds upon both Gram-positive bacteria; in any case, *S. epidermidis* yielded the lowest resistance among all six microorganisms considered. The results obtained for *C. albicans* were rather similar to those for *S. aureus*, although a relatively stronger action by medium and high MW chitosans could be perceived.

When impregnated into the cotton fabric, the results pertaining to the same time period were quite different from those observed in the culture medium. In the case of incubation in cotton fabric for 1 h, and regardless of the microorganism tested, a stronger activity was always obtained at higher MW. This difference was most evident for Gram-negative bacteria, since it means a reversed trend compared with the dependence of the antibacterial activity on MW (as reported previously). In the particular cases of *K. pneumoniae* and *E. coli*, high and medium MW chitosans presented results similar to those attained in culture medium, thus suggesting that incubation in a different matrix does not affect the activity of these compounds; however, the antibacterial activity of COS against both bacteria, and of low MW chitosan, only against *K. pneumoniae*, were much weaker (*ca.* one-third in the case of COS). Once again, the antibacterial action over *P. aeruginosa* was negligible, especially for COS. Regarding both cocci, chitosans also proved more effective: the three chitosans exerted an even higher activity when in the cotton fabric, whereas COS mixtures lost efficacy relative to the culture medium. The results encompassing *C. albicans* were relatively similar to those encompassing *S. aureus*, although the antifungal action in the cotton fabric was more evident for COS and low MW chitosan (*ca.* 2-fold higher than those observed in culture medium).

By 4 h, some differences relative to 1 h were observed: the COS effect over the 3 Gram-negative bacteria tested (*E. coli*, *K. pneumoniae*, and *P. aeruginosa*), when incubated in cotton fabric, appeared to be stronger than that observed with chitosans; the action of chitosans over the two Gram-positive bacteria (*S. aureus* and *S. epidermidis*) was stronger in culture medium than in cotton fabric. *Klebsiella pneumoniae* and *E. coli* again presented higher sensitivity at lower MW. Furthermore, the initial inocula of both

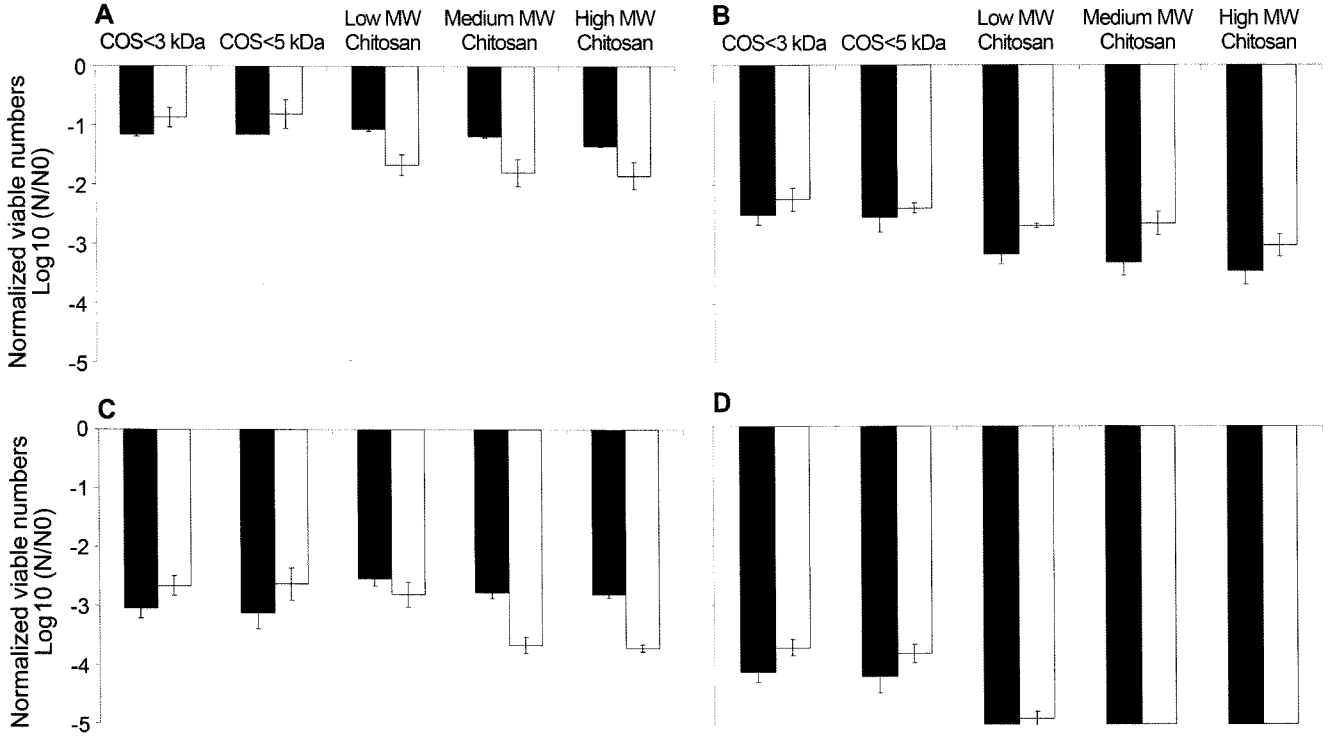

Fig. 2. Effects (average±standard deviation) of different MW chitosans and COS upon two Gram-positive bacteria [*S. aureus* after 1 (**A**) and 4 h (**B**), and *S. epidermidis* after 1 (**C**) and 4 h (**D**)] incubated in Müller–Hinton broth (■) or cotton fabric (□).

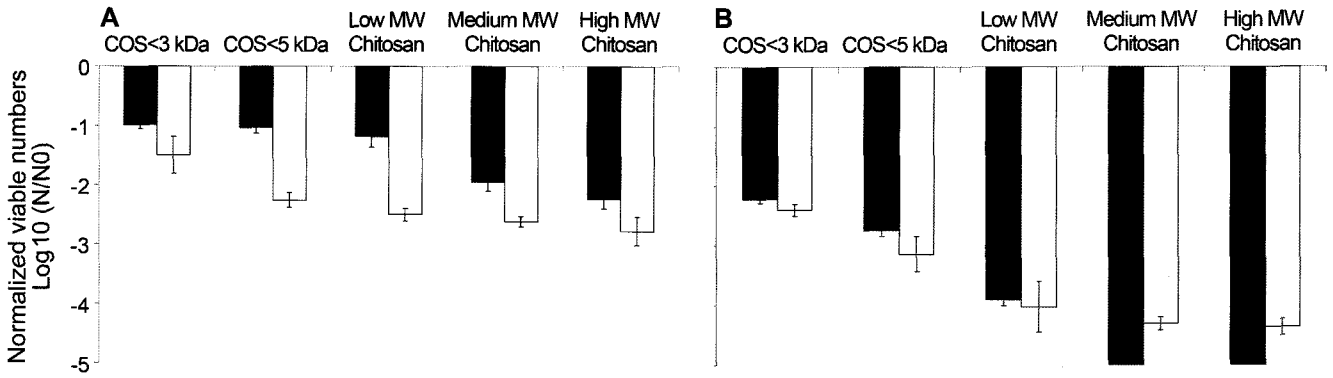

Fig. 3. Effects (average±standard deviation) of different MW chitosans and COS upon *Candida albicans*, after 1 (**A**) and 4 h (**B**), incubated in Müller–Hinton broth (■) or cotton fabric (□).

bacteria (10^5 CFU/ml) was depleted in the presence of either COS mixture and low MW chitosan in the culture medium, whereas a reduction of *ca.* 3-log cycles was achieved by both COS mixtures when applied onto the cotton fabric. It has been claimed [7] that 0.10% (w/v) of a 28.89 kDa chitosan impregnated onto cotton fabric would be enough to reduce the initial population (10^7 CFU/ml) of *E. coli* by 60%. At this time, it was noted that application of said compounds onto cotton fabric affected more the action of COS mixtures and low MW chitosan over Gram-negative bacteria, as they lost almost half of their antibacterial potential. In addition, the action of all compounds upon *P. aeruginosa* remained almost unnoticed.

Regarding either Gram-positive bacterium, an increase in antibacterial activity was seen once again along with an increase in MW, both in the culture medium and in the fabric; this trend was also suggested elsewhere [30], with a higher antibacterial effect by a high MW chitosan (*ca.* 600 kDa) when compared with a low MW chitosan (*ca.* 150 kDa), over *S. aureus* and *S. epidermidis* upon impregnation onto cotton. *Staphylococcus epidermidis* also showed a high susceptibility, irrespective of the matrix, to the 3 chitosans at stake: its viable numbers decreased to nondetectable levels in all cases. Similar results were again obtained towards *C. albicans.* However, in the case of COS mixtures, an apparent higher effect on the fabric (between 2.6- and 3.2-log cycle reduction) than in the culture medium (*ca.* 2.2- to 2.4-log cycle reductions) was observed.

The results encompassing all five bacteria showed a clear tendency: the antimicrobial effect is strongly dependent on the type of target microorganism (Gram-negative *versus* -positive) and on the MW of chitosan (higher at lower MW, in the case of Gram-negative bacteria, and the reverse in the case of Gram-positive bacteria). This conclusion is consistent with claims by other researchers [22, 41]; the apparent differential action upon Gram-positive and -negative microorganisms has been suggested [42] to probably result from the intrinsic difference in their cell wall structure, as 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 was indeed confirmed that COS actually penetrate *E. coli* cells, and hence suggesting that its antibacterial activity is chiefly caused by inhibition of DNA transcription [20]. Furthermore, a strong antifungal effect of chitosans was detectable upon *C. albicans*, which

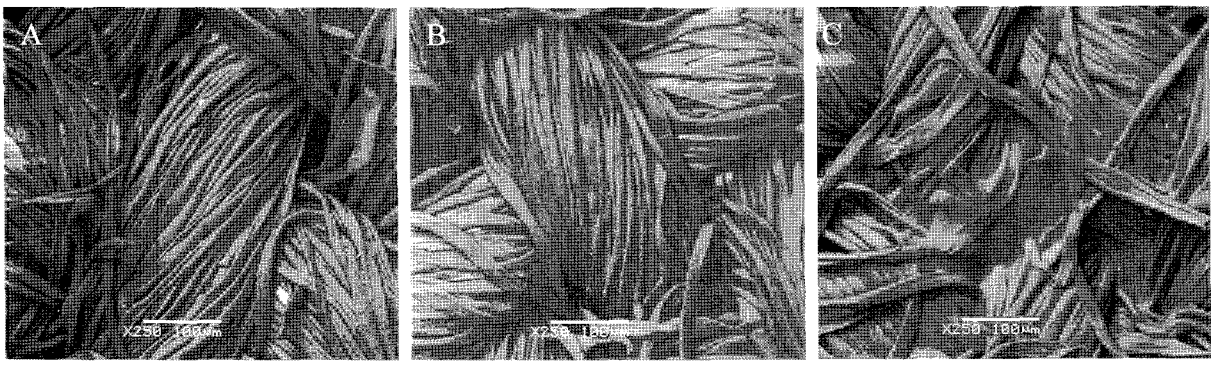

Fig. 4. Scanning electronic microscopy images of cotton fabric (**A**); cotton fabric with <3 kDa COS at 0.5 mg/ml (**B**); and cotton fabric with high MW chitosan at 0.5 mg/ml (**C**).

resembles the action over Gram-positive bacteria and thus suggesting a similar mechanism of action of chitosans over it. In the case of *P. aeruginosa,* the much lower outer-membrane permeability, which distinguishes it from other Gram-negative bacteria, strongly reduces the antimicrobial activity of COS, relative to those on *K. pneumoniae* and *E. coli* [8, 21].

Even though the overall trend in solution and in the fabric is virtually the same, the modes of action of chitosan and COS are probably different, since in solution, the action by oligosaccharides is faster than by higher MW chitosans. On the contrary, chitosans seemed to work much faster than COS when impregnated into cotton.

In fact, our results suggest that COS may actually lose some efficacy. This may result from the faster contact of bacteria with chitosan, which owing to their higher MW (and resulting higher viscosity) probably do not penetrate so deep into the fabric, hence remaining essentially on the surface. This possibility was in fact corroborated by our SEM images (Fig. 4), which showed a surface similarity between the control and the COS samples (Fig. 4A and 4B), whereas the impregnation with chitosans led to a topography change on the surface (Fig. 4C). These changes indicate the presence of chitosans on the cotton surface owing to their viscosity, originating a kind of layer that covers the cotton fibers and fills the spaces between them. Regarding COS, their lower MW (and consequent higher diffusion into the cotton matrix) may lead the efficacy of these compounds to be dependent on the incursion of bacteria also into the cotton disk core, so the presence of the microorganisms on the surface will not suffice. Regarding that most skin disorders are recurrent infections by its own microflora (mostly Gram-positive), as in atopic dermatitis or psoriasis, or even candidiasis (resulting from cutaneous infection with *Candida albicans*) [5, 9, 31], our results suggests that high MW chitosan is the most appropriate compound for use in future studies concerning functional textiles, in view of its widest spectrum of action, with special incidence over Gram-positive bacteria.

Acknowledgments

Funding for Author J. C. Fernandes was *via* a PhD fellowship, administered by Fundação para a Ciência e a Tecnologia (Ref. SFRH/BD/31087/2006), and supervised by Author F. X. Malcata.

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