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Original Article

Antimicrobial activity of shrimp chitin and chitosan from different treatments and applications of fish preservation

GUO-JANE TSAI,* WEN-HUEY SU, HSING-CHEN CHEN AND CHORNG-LAING PAN

Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan 202

ABSTRACT: The effects of the deacetylation degree (DD) and preparation methods for chitosan on antimicrobial activity were evaluated. Chemically prepared chitin (CH-chitin) and microbiologically prepared chitin (MO-chitin) were obtained from shrimp shells. The CH-chitin and MO-chitin were further chemically deacetylated to obtain various chitosan products of which their DD ranged from low (47–53%) through medium DD (74–76%) to high (95–98%). In addition, MO-chitin was deacetylated also by various proteases. The antimicrobial activities of these products were evaluated in medium with pH 6.0. Neither the CH-chitin, MO-chitin nor protease-deacetylated chitinous products showed any antimicrobial activity. For chitosan, antimicrobial activity increased with increasing DD, and was stronger against bacteria than against fungi. The minimal lethal concentrations (MLC) of chitosan with a high DD against *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Vibrio cholerae*, and *V. parahaemolyticus* were all in the range of 50–200 p.p.m., whereas the MLC against *Candida albicans* and *Fusarium oxysporum* were 200 p.p.m. and 500 p.p.m., respectively. No antifungal activity was found at 2000 p.p.m. against *Aspergillus fumigatus* or *A. parasiticus*. Pretreatment of fish fillets (*Oncorhynchus nereka*) with 1% chitosan solution (high DD) for 3 h retarded the increase in the volatile basic nitrogen content, as well as the counts for mesophiles, psychrotrophs, coliforms, *Aeromonas spp*., and *Vibrio* spp. The shelf life was consequently extended from 5 days to 9 days.

KEY WORDS: **antimicrobial activity, bacteria, chitin, chitosan, fish preservation, fungi, shrimp shell.**

INTRODUCTION

Chitosan, a partially deacetylated chitin [poly-β-(1→4)*N*-acetyl-D-glucosamine] can activate many plant's defense responses by eliciting the synthesis of phytoalexins, cell wall phenols, and callose.[1–6] It can also directly inhibit the growth of a wide range of fungi[7–13] and bacteria.[13,14–17] The fungicidal and bactericidal action of chitosan appears to be mediated by the electrostatic forces between the protonated NH_2 group in chitosan and the negative residues at cell surfaces.[2,18–19] The number of protonated NH_2 groups present in chitosan increases with increased degree of deacetylation (DD). Therefore, the DD for chitosan influences antimicrobial activity.

Chitosan is usually manufactured from crustacean shells (crabs, shrimps, and crayfishes) either by chemical or microbiological treatments. In a typical chemical process, NaOH, HCl and a de-coloring agent are used sequentially to remove the residual proteins, calcium and color from crustacean shell to obtain chitin, and the chitin is then deacetylated further with sodium hydroxide to obtain chitosan.[20] In a typical microbiological preparation of chitin, a proteolytic bacterium, *Pseudomonas maltophilia* LC 102 is used to remove proteins from shrimp shell,[21] and the chitin deacetylase from *Mucor rouxii*,[22–24] *Abisidia butleri*[25] or *Aspergillus nidulans*[26] can then be used

*Corresponding author: Tel: 886-2-2462-2192 ext. 5139. Fax: 886-2-2462-4113. Email: tsaigj@wfd.ntou.edu.tw
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to convert the chitin to chitosan. These different preparation methods are likely to result in differences in the DD, the distribution of acetyl groups, the chain length, and the conformational structure of chitin and the chitosan molecule, all of which will affect the characteristics of chitin and chitosan.[27] In the present study, we therefore investigate and compare the antimicrobial activities of chitin and chitosan obtained by chemical and biological treatments of shrimp shell. Evidence of how the DD and the size of the chitin and chitosan molecules affects antimicrobial activity is presented, and chitosan's potential for the preservation of fish fillets is also demonstrated.

MATERIALS AND METHODS

Chemical preparation of chitin and chitosan

Chitin was isolated from shrimp (*Solenocera melontho*) shell waste by sequential treatments with 2.5 N NaOH and 2 N HCl.[18] This chemically treated chitin (CH-chitin) and the microbiologically treated chitin (described later) were deacetylated with 40% NaOH (1 g of chitin per 13 mL of NaOH) at 100°C for 1 h and 2.5 h to obtain low DD chitosan (i.e. chitosan with a low degree of deacetylation) and medium DD chitosan, respectively. High DD chitosan was obtained by deacetylation with 50% NaOH at 140°C in an oil bath for 3 h. The obtained residue (chitosan) was washed thoroughly with deionized water until the effluent became neutral, and was then dried in an oven at 70°C for 20 h. The degree of deacetylation of chitin and chitosan were measured by infrared spectroscopy[28] and a colloid titration method,[29] respectively. The molecular weight of chitin and chitosan was analyzed by capillary viscometry.[30]

Microbiological preparation of chitin

Chitin was microbiologically prepared according to the methods described by Chen and Hsu.[31] Briefly, shrimp shells were treated with 2 N HCl for 2 h for decalcification. The decalcified shells were washed using tap water until the pH of the drained water became neutral. Then, 5 kg of the treated shells were mixed with 45 L of basal medium (0.38% w/v K_2HPO_4, pH 8.5) in a fermentor (100 L; CMF-100; Chin Chi Trading Co., Taipei, Taiwan), and this mixture was sterilized (121°C, 60 min) and allowed to cool. The fermentor was then inoculated with 5 L of a culture (2.5×10^7 c.f.u./mL) of *Pseudomonas maltophilia* NO 1-1. This bacterium, which is isolated from soil, has a strong protease activity and no chitinase activity,[31] and it had already been cultured twice previously in nutrient broth (NB; Difco Laboratories, Detroit, MI, USA) at 30°C for 20 h. After fermentation at 30°C (air flow rate 4.5 L/min, stirring rate 160 r.p.m., pH 8.4) for 3 days and centrifugation (5000 ×*g* for 20 min), the chitin residue was washed with deionized water until the effluent became neutral, and was then dried in an oven at 70°C for 20 h. In the present report, this microbiologically prepared chitin is designated as MO-chitin.

Enzymatic preparation of chitosan from microbiologically prepared chitin

To 250 mL flasks containing 50 mL of MO-chitin suspension (0.5 g in 50 mL of 50 mM phosphate buffer, pH 7.0), 10 mL of various protease solutions (chymotrypsin, pepsin, pronase, and trypsin, which were obtained from Boehringer Mannheim Biochemicals, Mannheim, Germany) were added so that the final enzyme concentration was adjusted to 3.33 mg/mL. After reacting at 37°C in a water bath with reciprocal shaking for 24 h and then centrifugation (12000 ×*g* for 20 min), the residue was washed four times with deionized water and dried (70°C, 20 h).

Cultures for antimicrobial tests

Aeromonas hydrophila CCRC 13881, *Bacillus cereus* CCRC 10250, *Escherichia coli* CCRC 10674, *Pseudomonas aeruginosa* CCRC 10944, *Salmonella typhimurium* CCRC 10746, *Shigella dysenteriae* CCRC 13983, *Staphylococcus aureus* CCRC 12652, *Vibrio cholerae* CCRC 13860, and *V. parahaemolyticus* CCRC 10806 were purchased from the Culture Collection and Research Center (CCRC, Hsinchu, Taiwan). *Aeromonas hydrophila* YM1 was isolated from a hospital patient with diarrhea (kindly provided by Dr WC Tsai, National Yang-Ming University, Taiwan), and *Listeria monocytogenes* LM-LM was originally isolated from a contaminated store-bought chicken (kindly provided by Dr SC Chen, National Taiwan Ocean University, Taiwan). All the tested bacteria were primarily stored in NB (or NB with 3% w/v NaCl for *V. parahaemolyticus*) containing 50% sterile glycerol at –70°C. These bacterial strains were subcultured twice in NB (or NB with 3% w/v NaCl for *V. parahaemolyticus*) and incubated at 37°C (or 28°C for *A. hydrophila* CCRC 13881 and YM1) for 1 day for the antibacterial test of chitin and chitosan.

Fungal strains of *Aspergillus fumigatus* CCRC 30502, *Aspergillus parasiticus* CCRC 30117,

Candida albicans CCRC 20511, and *Fusarium oxysporium* CCRC 32121 were obtained from CCRC and stored on slants of potato dextrose agar (PDA, Difco) at 4°C. The yeast *C. albicans* was subcultured twice in yeast extract-malt broth (YM broth; Difco) at 25°C for 2 days. The mold strains of *F. oxysporium*, *A. fumigatus*, and *A. parasiticus* were subcultured once on the PDA slants at 25°C for 7 days, and spores were washed from the surface with 10 mL of sterile deionized water containing 0.05% w/v Tween 20 (Sigma Chemical Co., St Louis, MO, USA) for the antifungal test of chitin and chitosan.

Antimicrobial tests

One percent of sterile chitin suspension or chitosan solution in 0.1 N HCl was prepared as the stock solution by adding 1 g of chitin (or chitosan) to 50 mL of deionized water, followed by sterilization at 121°C for 15 min and then the addition of 50 mL of sterile 0.2 N HCl. To 50 mL flasks containing 10 mL NB (or NB plus 3% NaCl for *V. parahaemolyticus*), various volumes of chitin (or chitosan) stock solution were added and the pH value was adjusted to 6.0. A volume of 100 μL of each tested bacterium ($\approx 10^8$ c.f.u./mL) was inoculated into the flasks. After incubation with shaking (120 r.p.m.) at 37°C (or 28°C for *A. hydrophila* CCRC 13881 and YM1) for 2 days, duplicate samples of 0.1 mL of decimal dilutions were spread on nutrient agar (NA) plates (or plates with NA plus 3% NaCl for *V. parahaemolyticus*). After incubation at 37°C (or 28°C for *A. hydrophila* CCRC 13881 and YM1) for 2 days, the colonies on the plates were counted.

For the antifungal test, the same protocol was used except that 20 mL of YM broth was added to the 125 mL flasks. After inoculation of 0.1 mL of either a yeast culture ($\approx 10^6$ c.f.u./mL) of *C. albicans* or the spore suspension ($\approx 10^6$ spores/mL) of *F. oxysporium*, *A. fumigatus*, and *A. parasiticus*, and incubation at 25°C for 2 days (yeast) or 7 days (molds), the fungal growth in YM broth was measured by either counting the number of colonies on the YM agar plates (yeast) or weighing the mycelium (mold). For mycelium mass measurement, the mold culture was filtered through a preweighed filter (Whatman no. 1). The mycelia on the filter were then washed five times with deionized water, dried in an oven at 100°C for 24 h, and then weighed. The experiment was run in duplicate. The minimal lethal concentration (MLC) was defined as the lowest concentration at which no colony was detected on the plate for the replicate samples.

Storage test of fish fillet presoaked in chitosan

Samples (10 g each) of freeze-thawed salmon fillets (*Oncorhynchus nereka*), which were purchased from the local market and cut aseptically into small pieces (2.5 cm × 2 cm× 1 cm) in the laboratory, were soaked in various concentrations of chitosan solutions (0.2%, 0.5%, or 1.0% in 0.1 N HCl, adjusted to pH 6.0 with 1 N NaOH) for 3 h, and then drip-dried (5 min). These treated samples were placed in Petri dishes, covered with polyethylene film and stored at 4°C. The same protocol was used for the control, except that the fish fillets were soaked in 0.1 N HCl (adjusted to pH 6.0 with 1 N NaOH) to which chitosan had not been added. Four samples from each treatment were removed at intervals. In two of these samples, the volatile basic nitrogen (VBN) was measured using the microdiffusion method.[32] The other two samples were blended for 1 min using a Stomacher (Seward Stomacher 400; London, UK) after adding 90 mL of 0.1% peptone water, and then subjected to microbiological evaluation and pH measurement. Following the method described by Chen *et al.*,[14] decimal dilutions (100 μL) were spread in duplicate on various media and incubated as follows: aerobic mesophilic count, plate count agar (PCA; Difco) at 30°C for 48 h; psychrotrophic count, PCA at 7°C for 10 days; coliform count, violet red bile agar (VRBA; Difco) at 37°C for 24 h; *Aeromonas* count, starch ampicillin agar[33] at 28°C for 24 h; *Pseudomonas* count, *Pseudomonas* isolation agar (PI; Difco) at 26°C for 24 h; and *Vibrio* count, thiosulfate-citrate–bile–sucrose agar (TCBS; Difco) at 26°C for 24 h.

RESULTS AND DISCUSSION

The degrees of deacetylation (DD) and the molecular weights of various chitin and chitosan preparations are shown in Table 1. The molecular weights of CH-chitin and MO-chitin could not be measured because for molecular weight analysis by capillary viscometer, the chitin (or chitosan) needs to be dissolved in an acidic solution,[30] and both the CH-chitin and MO-chitin molecules were too large to be dissolved in 0.2 M acetic acid used here. However, as Table 1 shows, because the chitin molecules were degraded during alkaline deacetylation, the molecular weights of both CH-chitosan and MO-chitosan decreased with increasing DD. For example, the molecular weights for DD53, DD76 and DD98 CH-chitosan were 1.08×10^6, 2.85×10^5 and 4.91×10^4, respectively. In the present study, in an effort to reduce this degradation, different proteases were used to achieve uncon-

ventional deacetylation of MO-chitin. The DD of the chitinous products obtained from the digestion of the MO-chitin by pronase, pepsin, trypsin, and chymotrypsin were 63%, 62%, 60%, and 69%, respectively. During traditional alkaline deacetylation of chitin, the chitinous product becomes soluble in acidic aqueous solutions when its DD exceeds 50%, at which point it is called chitosan.[34] In the present study, however, although the DD of the protease-deacetylated chitinous products all exceeded 50%, they could not be dissolved in 0.2 M acetic acid and, in this respect, resembled the original MO-chitin more closely than the MO-chitosan.

Table 1 Degree of deacetylation and molecular weight of chitin and chitosan obtained from shrimp shell treated chemically or microbiologically

	Degree of deacetylation (%)	Molecular weight[d]
CH-chitin[a]	35 ± 5[c]	ND
CH-chitosan[b]	53 ± 4	1.08×10^6
	76 ± 5	2.85×10^5
	98 ± 3	4.91×10^4
MO-chitin[a]	32 ± 6	ND[c]
MO-chitosan[b]	47 ± 5	1.10×10^6
	74 ± 5	3.10×10^5
	95 ± 4	5.10×10^4

[a]CH-chitin, chemically prepared chitin; MO-chitin, microbiologically prepared chitin.
[b]CH-chitosan, chitosan from alkaline deacetylation of CH-chitin; MO-chitosan, chitosan from alkaline deacetylation of MO-chitin.
[c]Average ± SE ($n = 4$).
[d]Average of two measurements.
ND, not determined.

No antimicrobial activity was observed for CH-chitin, MO-chitin, or protease-deacetylated chitinous products (data not shown). The MLC of CH-chitosan and MO-chitosan with various DD are shown in Table 2. In general, the MLC for CH-chitosan and MO-chitosan were similar, and became smaller with increasing DD. With the exception of *A. hydrophila* and *S. typhimurium*, the MLC of chitosan with a high DD (DD95 and DD98 in Table 2) against Gram-negative bacteria (e.g. *E. coli*, *P. aeruginosa*, *S. dysenteriae*, *V. cholerae,* and *V. parahaemolyticus*) and Gram-positive bacteria (e.g *B. cereus*, *L. monocytogenes*, and *S. aureus*) were in the range of 50–200 p.p.m. In contrast, the antifungal activities of the tested chitosan products were lower. The MLC of the high DD chitosan products against *C. albicans* and *F. oxysporium* were 200 p.p.m. and 500 p.p.m., respectively. There was no antifungal activity observed against either *A. fumigatus* or *A. parasiticus* at 2000 p.p.m.

The antibacterial or antifungal activity of chitosan depends on the protonated positive charge number of chitosan and the number of the nega-

Table 2 Minimal lethal concentrations (MLC)of chitosan obtained from shrimp shell treated microbiologically or chemically against various microorganisms

	MLC (p.p.m.)					
Tested microorganisms	DD95 (MO)[a]	DD98 (CH)[b]	DD74 (MO)[a]	DD76 (CH)[b]	DD47 (MO)[a]	DD53 (CH)[b]
Bacteria, Gram negative						
Aeromonas hydrophila CCRC 13881	500	1000	1000	1000	1500	>2000
Aeromonas hydrophila YM1	500	500	500	500	>500	>500
Escherichia coli CCRC 10674	100	100	100	100	500	200
Pseudomonas aeruginosa CCRC 10944	200	150	>200	200	>200	>200
Salmonella typhimurium CCRC 10746	1500	1500	1500	1500	>2000	>2000
Shigella dysenteriae CCRC 13983	200	200	>200	>200	>200	>200
Vibrio cholerae CCRC 13860	150	200	200	200	>200	>200
Vibrio parahaemolyticus CCRC 10806	100	100	150	100	>150	>150
Bacteria, Gram positive						
Bacillus cereus CCRC 10250	200	200	1000	500	1000	1000
Listeria monocytogenes LM-LM	100	150	150	150	150	150
Staphylococcus aureus CCRC 12652	100	50	100	100	100	150
Fungi						
Candida albicans CCRC 20511	200	200	500	800	800	800
Fusarium oxysporum CCRC 32121	500	500	1000	500	>2000	>2000
Aspergillus fumigatus CCRC 30502	>2000	>2000	>2000	>2000	>2000	>2000
Aspergillus parasiticus CCRC 30117	>2000	>2000	>2000	>2000	>2000	>2000

[a]DD95 (MO), DD74 (MO), DD47 (MO): 95%, 74%, or 47% deacetylated chitosan from microbiologically prepared chitin.
[b]DD98 (CH), DD76 (CH), DD53 (CH): 98%, 76%, or 53% deacetylated chitosan from chemically prepared chitin.

tive charge on the microbial surface.[12,18–19] Therefore, a chitosan with a higher DD, which has a higher positive charge, would be expected to have a stronger antimicrobial activity. As well as DD, the size and conformation of the chitosan molecule might also affect its antimicrobial activity. During the conventional alkaline deacetylation of chitin at high temperatures, both the molecular size and the DD of the chitosan obtained changed with the intensity of the deacetylation reaction, as shown in Table 1. In addition, during the alkaline deacetylation process, the conformational transition is already known to occur; that is, the chitin molecule gradually loses its crystallinity[35] and becomes more extended.[30] Therefore, when investigating the effect of DD on the antibacterial function of chitosan, as shown in Table 2, we can not exclude the influential factors of size and conformation on the chitosan molecule. Therefore, different proteases in mild conditions (pH 7.0, 30°C) were used to deacetylate chitin without causing either any degradation or conformational changes to the chitin molecule. The fact that the protease-deacetylated chitinous products with a DD in the range of 60–69% did not have any antimicrobial activity, which was apparently different from the high activity of DD69 chitosan prepared from the conventional alkaline deacetylation of shrimp chitin,[14] supports our previous hypothesis about the effects of a chitosan molecule's size and conformation on its antibacterial activity.

Figure 1 shows the changes in the mesophilic and psychrotrophic counts, the VBN contents, and the pH values of fish fillets that were presoaked in

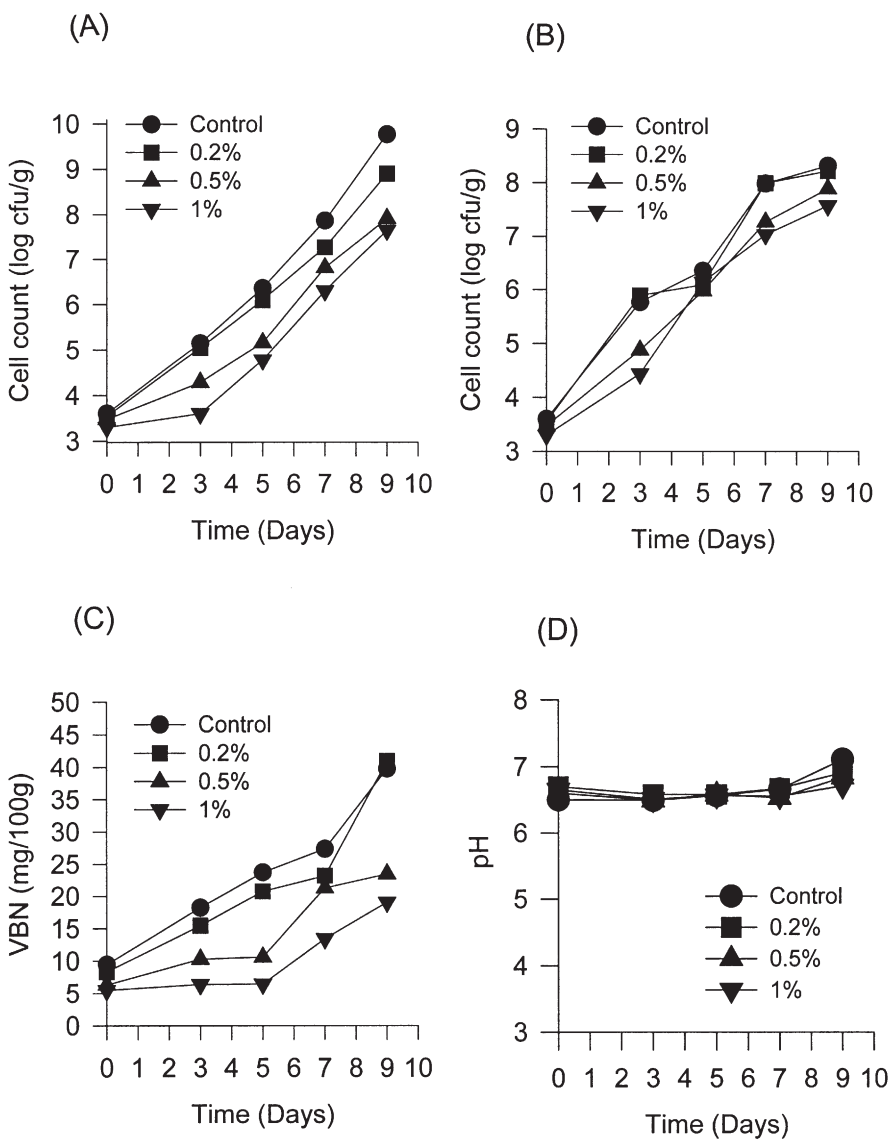

Fig. 1 Changes in (a) mesophilic count; (b) psychotropic count; (c) volatile basic nitrogen; and (d) pH value of *Oncorhynchus nereka* presoaked in various concentrations of chitosan solutions for 3 h, followed by storage at 4°C.

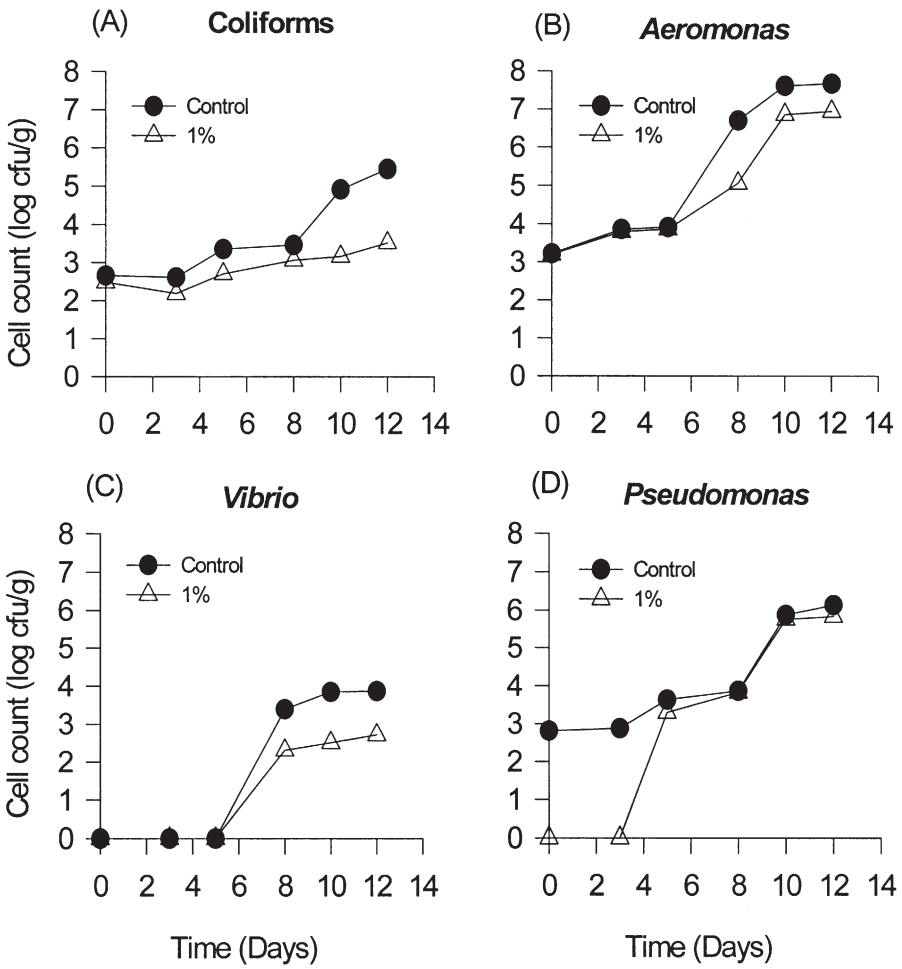

Fig. 2 Changes in cell counts of different microorganisms in *Oncorhynchus nereka* presoaked in 1% chitosan solution for 3 h, followed by storage at 4°C.

CH-chitosan (DD98) solution (0–1.0%) for 3 h, and then removed and stored at 4°C. Increases in the mesophilic and psychrotrophic counts and in the VBN content of fish fillets were retarded when the concentration of chitosan solution used was increased. After 9 days of storage, the mesophilic and psychrotrophic counts in the samples treated with 1% chitosan were lower by approximately 2 log units and 1 log unit, respectively, compared to the control samples (Fig. 1a,b). The VBN content in the samples treated with 1% chitosan started to increase only after 5 days of storage and its VBN content was 18 mg/100 g by the ninth day, whereas the VBN content for the control increased steadily and, by the ninth day, its value was 40 mg/100 g (Fig. 1c). Although the pH values for the control and experimental samples were similar after 5 days (at ≈ pH 6.5), they increased to pH 7.0 and pH 6.6, respectively, after 9 days of storage (Fig. 1d). The control samples started to develop a putrid odor after 5 days of storage, and samples treated with 1% chitosan did the same after 9 days.

Chitosan pretreatment also effectively inhibited the growth of coliforms in the fish fillets (Fig. 2a). After 12 days of storage, the counts for *Aeromonas* species and *Vibrio* species were likewise reduced in the experimental samples compared to those in the control (Fig. 2b,c). Although the *Pseudomonas* counts for the control and experimental samples were similar after 12 days, the growth of *Pseudomonas* species was effectively inhibited within the first 3 days of storage (Fig. 2d).

In conclusion, the antimicrobial activities of both chemically prepared and microbiologically prepared chitin/chitosan were similar, and in both cases the activity increased with increasing DD. As well as DD, the size and conformational extension of the chitin and chitosan molecules also appears to be critical for their antimicrobial function. Chitosan generally has a stronger activity against bacteria rather than against fungi. Chitosan with a high DD (DD98) effectively inhibited various bacteria and, therefore, showed potential for extending the shelf life of refrigerated fish fillets.

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