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Chitosan: antimicrobial activity, interactions with food components and applicability as a coating on fruit and vegetables

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Abstract

Chitosan has recently gained more interest due to its applications in food and pharmaceutics. Among others, the antimicrobial activity of chitosan has been pointed out as one of its most interesting properties of chitosan.

The aim of this study was threefold: (1) the quantification of the antimicrobial effect of chitosan with a deacetylation degree of 94% and a molecular weight of 43 kDa on different psychrotrophic spoilage organisms and food pathogens. (2) The determination of the influence of different food components (starch, whey protein, NaCl and oil) on the antimicrobial effect of chitosan and (3) the investigation of the effects of chitosan coatings on controlling decay of minimally processed fruits and vegetables (strawberry and lettuce). For the first aim several bacteria and yeast were exposed to chitosan concentrations varying from 40 to 750 mg/l. Generally, Gram-negative bacteria seemed to be very sensitive for the applied chitosan (MIC $\leqslant$ 0.006% (w/v)) while the sensitivity of Gram-positive bacteria was highly variable and that of yeast was intermediary (0.01% (w/v)). To achieve the second aim, the media, with one of these components added, were inoculated with *Candida lambica* ($\pm$2 log cfu/ml) and were incubated at 7°C until the yeast reached the stationary phase. Starch, whey proteins and NaCl had a negative effect on the antimicrobial activity. Oil conversely had no influence. For the third aim, the chitosan coating was formed by dipping the products in a chitosan–lactic acid/Na-lactate solution from which the pH was adjusted to the pH of the products. These products were equilibrium modified atmosphere (EMA)-packaged, stored at 7°C and during storage sensorially and microbiologically evaluated. A chitosan coating on strawberries was applicable while on mixed lettuce the chitosan coating was not applicable due to the development of a bitter taste. The microbiological load on the chitosan-dipped samples was lower for both products. The antimicrobial effect of chitosan on lettuce disappeared after 4 days of storage, while it maintained on the strawberries during 12 days.

Keywords: Chitosan; Antimicrobial coating; Fruit and vegetables

1. Introduction

Chitosan (poly β-(1$\rightarrow$4)*N*-acetyl-D-glucosamine), a deacetylated form of chitin, is a natural antimicrobial compound. On the one hand it can be obtained from crustacean shells (crabs, shrimp and crayfishes) either by chemical or microbiological processes and on the other hand it can be produced by some fungi (*Aspergillus niger*, *Mucor rouxii*, *Penecillium notatum*) (Tan et al., 1996; Knorr, 1984).

These variations in preparation methods are likely to result in differences in the deacetylation degree, the distribution of acetyl groups, the chain length and the conformational structure of chitosan (Tsai et al., 2002) and will thereby have an influence on the solubility, the antimicrobial activity and other properties. Next to chitosan itself, several chitosan derivatives are known for their antimicrobial activity, e.g. acid-free-water-soluble chitosan (Ilyina et al., 2000), quaternary *N*-alkylchitosan (Jia, 2001), sulfonated chitosan (Chen et al., 1998) and *N*-carboxybutyl chitosan (Muzzarelli et al., 1990). The applications of chitosan and its derivatives are widespread, they are used in agriculture, medicine, environment, food, etc. Apart from its antimicrobial effect, chitosan is also used in food as (1) clarifying agent in apple juice (Boguslawski et al., 1990; Root and Johnson, 1978; Soto-Peralta et al., 1989), (2) antioxidant in sausages (Xie et al., 2001) (3) enzymatic browning inhibitor in apple and pear juices

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(Sapers, 1992) and in potatoes (Dörnenburg and Knorr, 1997). Chitosan can also be used as an antimicrobial film to cover fresh fruits and vegetables (Du et al., 1997; El-Ghaouth et al., 1991a, 1992; Jiang and Li, 2001; Kittur et al., 2001; Li and Yu, 2001; Zhang and Quantick, 1998). Chitosan activates several defense processes in the host tissue (El-Ghaouth et al., 1992), acts as a water-binding agent and inhibits various enzymes (Young et al., 1982).

The antimicrobial activity of chitosan will depend on several factors such as the kind of chitosan (deacetylation degree, molecular weight) used, the pH of the medium, the temperature, the presence of several food components, etc. The mechanism of the antimicrobial activity has not been fully elucidated yet, but several hypotheses have been postulated. The most feasible hypothesis is a change in cell permeability due to interactions between the polycationic chitosan and the electronegative charges on the cell surfaces. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents (Papineau et al., 1991; Sudarshan et al., 1992; Fang et al., 1994; Chen et al., 1998; Young et al., 1982). Other mechanisms mentioned in the literature are the interaction of diffused hydrolysis products with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis (Hadwiger et al., 1985; Sudarshan et al., 1992) and the chelation of metals, spore elements and essential nutrients (Cuero et al., 1991).

In this study the antimicrobial effect of a commercial chitosan, with a high deacetylation degree (94%) and a low molecular weight (43 kDa) was screened against several psychrotrophic foodborne pathogens and spoilage micro-organisms and compared to those known from literature. Also the influence of different food components (oil, starch, whey proteins and NaCl) on the antimicrobial effect of chitosan was investigated. Finally, an evaluation of the applicability of the commercial chitosan as a food preservative for EMA-packed strawberries and lettuce was investigated.

2. Material and methods

2.1. Chitosan

Chitoclear^{TM} batch TM999 (Primex, Norway), produced from shrimp shells by chemical processes only (deproteination, deacetylation and depolymerization) and having a molecular weight of $\pm$43 kDa and a deacetylation degree of 94%, was used.

2.2. Susceptibility of psychrotrophic food pathogens and spoilage organisms

The micro-organisms used in this study are summarized in Table 1 together with their origin, optimal growth temperature and cultivation medium. The cultures were statically incubated overnight at their optimal temperature in their specific growth medium (liquid). Occurrence of growth at 7°C in the same media with different chitosan concentrations was followed during 60 days by visual detection (turbidity). Therefore, different chitosan concentrations ranging from 40 to 750 mg/l were added to the specific growth medium. A stock solution of 5000 mg/l chitosan was made in 2.5% (v/v) acetic acid (made from 100% acetic acid solution (Vel 90010, Merck Eurolab, Leuven, Belgium)), adjusted to the respective pH (5.0 or 5.5) by NaOH pellets (Vel 8688) and filter sterilized (Schleicher & Schuell, ∅ 0.45 μm, Dassel, Germany). The stock solution was diluted with sterile 2.5% (v/v) acetic acid with the same pH to concentrations of 100, 200, 400, 600, 800, 1000, 2000, 3000 and 4000 mg/l. Afterwards these solutions were diluted 10 times with one of the media, which was first heat sterilized. Because the

Table 1
Summary of the used strains together with their collection number or origin, optimal cultivation temperature and growth medium

Strain	Number	Origin	Cultivation temperature (°C)	Growth medium (Brand)
Candida lambica[a]	194	Mixed lettuce	30	Sabouraud (Oxoid, CM147)
Cryptococcus humicolus[a]	192	Mixed lettuce	30	Sabouraud (Oxoid, CM147)
Photobacterium phosphoreum	LMG 4233		30	MRS (Oxoid, CM361)
Pseudomonas fluorescens	LMG 1794	Prefilter water-works tanks	30	BHI (Oxoid, CM225)
Enterobacter aerogenes	LMG 2094		37	BHI (Oxoid, CM225)
Bacillus cereus	LMG 6924		30	BHI (Oxoid, CM225)
Brochothrix thermosphacta[a]	229	Ham	30	BHI (Oxoid, CM225)
Listeria monocytogenes	LMG 13305	Soft cheese	30	BHI (Oxoid, CM225)
Lactobacillus sakei subsp. *carnosum*[a]	216	Cooked ham	30	MRS (Oxoid, CM361)
Lactobacillus plantarum 1	LMG 6907	Pickled cabbage	30	MRS (Oxoid, CM361)
Lactobacillus plantarum 2[a]	530	Starterculture (dry sausages)	30	MRS (Oxoid, CM361)
Lactobacillus curvatus[a]	654	Biopâté	30	MRS (Oxoid, CM361)
Pediococcus acidilactici	LMG 6411	Starterculture	30	BHI (Oxoid, CM225)

[a] Isolated at the Laboratory of Food Microbiology and Food Preservation, Ghent University.

Table 2
Time for turbidity (days) at 7°C for different micro-organisms in traditional growth media with different chitosan concentrations at 7°C

Micro-organism	Chitosan concentration (mg/l)											
	pH	0	40	60	80	100	200	250	300	400	500	750
Candida lambica	5.5	5	5	5	5	6	14	ND[a]	40	—[b]	—	ND
Cryptococcus humiculus	4	12	12	12	—	—	—	ND	—	—	—	ND
Photobacterium phosphoreum	5.5	14	14	21	—	—	—	ND	—	—	—	ND
Pseudomonas fluorescens	5.5	8	18	28	—	—	—	ND	—	—	—	ND
Enterobacter aeromonas	5.5	39	47	—	—	—	—	ND	—	—	—	ND
Bacillus cereus	5.5	11	11	—	—	—	—	ND	—	—	—	ND
Brochothrix thermosphacta	5.5	5	5	5	—	—	—	ND	—	—	—	ND
Listeria monocytogenes	5.5	8	10	18	32	33	—	ND	—	—	—	ND
Lactobacillus sakei	5.5	4	4	4	4	6	12	ND	13	26	30	ND
Lactobacillus plantarum 1	5.0	24	ND	ND	ND	24	ND	49	ND	ND	—	—
Lactobacillus plantarum 2	5.0	23	ND	ND	ND	25	ND	25	ND	ND	25	—
Lactbacillus curvatus	5.0	22	ND	ND	ND	22	ND	22	ND	ND	ND	—
Pediococcus acidilactici	5.0	40	ND	ND	ND	40	ND	40	ND	ND	—	—

[a] ND: no data.
[b] No turbidity within 60 days.

concentration of acetic acid is the same in all samples, the differences in antimicrobial effect can only be caused by the activity of chitosan. The pH of the sterile media was adjusted with sterile HCl (Vel 1030) to the respective pH (Table 2). The media and chitosan solutions were aseptically mixed and centrifuged at 16,300*g* (Sorvall RC-58, Refrigerated Superspeed Centrifuge, Dupont Instruments) to remove eventually formed complexes (between chitosan and several components in the media) so that visual detection of growth was possible. Five milliliter of these solutions were finally inoculated with 100 µl of a microbial culture in the stationary phase to obtain an initial inoculation level of approximately 10^4 cfu/ml and stored at 7°C. The time, at which turbidity occurred, was registered. The MIC was defined as the minimal concentration at which no growth occurred during 60 days incubation at 7°C.

2.3. Interaction with food components

To estimate the effect of different food components on the antimicrobial activity of chitosan, the growth of *Candida lambica* was followed at 7°C in Sabouraud (Oxoid, CM147, Unipath, Basingstoke, Hampshire, UK) with varying chitosan concentrations (0, 50 and 100 mg/l) at pH 5.0 and with the separate addition of the following food components: starch (0%, 1% or 30% water soluble starch, Difco; Becton Dickinson, Sparks, USA), proteins (0%, 1% or 10% whey protein isolate (iso-electric point (IEP) 5.0–5.2) containing 85% of β-lactoglobulin and 15% of α-lactalbumin (Davisco, France)), oil (0%, 1% or 10% sunflower oil, Derby + 1 ml Tween 80 (Merck, Schuchart, Hohenbrunn, Germany)) and NaCl (0%, 0.5% and 2% NaCl (Vel 1723)). Only the experiments with proteins were performed at two different pH values (4.0 and 6.0).

To investigate the effect of pH on the antimicrobial activity of chitosan, experiments were performed with pure Sabouraud medium (Oxoid, CM147) from which the pH was adjusted to 4.0 and 6.0 with, respectively, 1 N HCl (Vel 1030) and a 1 N NaOH solution (made from NaOH-pellets, Vel 8688).

For the experiment with starch, the chitosan-medium solutions were prepared in the respective concentrations and added to the different amounts of starches. This mix was autoclaved and the pH was adjusted with sterile NaOH (1 N) to 5.0. In the experiment with proteins, the whey protein isolate was autoclaved dry and separately from the chitosan-medium solutions to avoid denaturation of the protein. After autoclaving and cooling, the chitosan-medium solutions were aseptically added to the proteins and the pH was adjusted to 4.0 or 6.0 with, respectively, sterile 1 N HCl- (Vel 1030) or 1 N NaOH-solution (made from NaOH- pellets, Vel 8688) (1 N). For the experiment with NaCl, the various chitosan-medium solutions were made and autoclaved. After autoclaving, the pH of the solutions was adjusted. To make the oil-chitosan-medium solutions, the oil was autoclaved separately, afterwards aseptically added to the autoclaved and pH-adjusted chitosan-medium solution, and mixed well with a sterile hand blender (Moulinex Spirali, DG3, Paris, France). Filter-sterilized Tween 80 (Merck) (1 ml) was added to facilitate the mixing and to stabilize the emulsion.

The different solutions were inoculated with approximately 2 log cfu/ml of *C. lambica* and incubated at 7°C. The number of *C. lambica* in each solution was determined by spread plating on a non-selective medium (Plate Count Agar (PCA, Oxoid, CM325)) followed by incubation of the plates for two days at 30°C. Growth parameters (lag phase λ (h) and maximum specific growth rate μ (h^{-1})) were estimated by fitting the data to

the growth equation of Baranyi and Roberts (1994) using SPSS 9.0 for Windows.

2.4. In situ experiments

Whole strawberries (J. Van Landschoot, Ghent, Belgium) and mixed lettuce (Allgro N.V., Sint-Lievens-Houtem, Belgium), consisting of 20% endive, 20% curled endive, 20% radicchio lettuce, 20% lollo rosso and 20% lollo bionta lettuce, were dipped in a 2% lactic acid/Na-lactate solutions (Debevere, 1988; Zeitoun and Debevere, 1990) or in the same lactic acid/Na-lactate solution in which 0.5% (w/v) chitosan was dissolved. Undipped samples were used as a control. The pH of the dipping solutions was adapted to the pH of the food product (3.7 for the strawberries and 5.0 for the mixed lettuce) with NaOH pellets (Vel 8688) and an HCl (1 N) (Vel 1030) solution in order to minimize taste deviations. After dipping, the strawberries and lettuce were dried at room temperature for 15 minutes and then packaged to obtain an equilibrium-modified atmosphere (3% O_2 and 7% CO_2) as described in Jacxsens et al. (1999). The respiration of the products was measured through closed system experiments by measuring the O_2 and CO_2 concentrations over time (Jacxsens et al., 2000). From these results the O_2 transmission rate of the specific packaging film was calculated. For the undipped and lactate-dipped strawberries the same film was used (WA 7805-1, Hyplast N.V. Klerck's Group, Hoogstraten, Belgium) with an O_2 transmission rate (OTR) of 2290 ml $O_2/(m^2\ 24\,h\ atm)$ at 7°C and 90% RH. For the strawberries and lettuce treated with chitosan and for the lettuce treated with the lactic acid/Na-lactate solution only, a film with higher OTR (3200 ml $O_2/(m^2$ 24 h atm) at 7°C and 90% RH) was used (WA 7805-3, Hyplast NV). For the untreated lettuce, a film with lower OTR (2021 ml $O_2/(m^2\,24\,h$ atm) at 7°C and 90% relative humidity) was necessary (WA 4060-6, Hyplast N.V.).

2.4.1. Analysis of the sensory quality

During storage at 7°C the products were sensorially evaluated (trained panel of 6 persons) in terms of their flavor, taste, texture, juiciness, color and general appearances. The first part (organoleptical characteristics such as taste, texture and juiciness) was conducted under red light to avoid interferences with visual judgment. The second part (visual characteristics like color, general appearances and general freshness) was conducted in daylight. The scores given by the panel varied from 1 (=fresh) to 10 (=deteriorated). A sample was considered as unacceptable for a sensorial characteristic if the score was higher than 5.

2.4.2. Microbiological analyses

Thirty grams of product was diluted tenfold with peptone saline solution (8.5 g/l NaCl (Vel 1723) + 1 g/l peptone (Oxoid, L34)). After homogenization in a stomacher (Led Techno, Stomacher Lab-Blender 400), dilution series were made and the appropriate dilutions were pour-plated on (1) Plate Count Agar (PCA, Oxoid CM325) for the aerobic psychrotrophic count, (2) deMann, Rogosa and Sharpe Agar (MRS, Oxoid, CM361) for lactic acid bacteria and spread-plated on (3) Yeast Glucose Chloramphenicol (YGC, Sanofi Diagnostics Pasteur, Marnes-La Coquette, France) for yeast and moulds. PCA plates were incubated 5 days at 22°C, MRS plates 3 days at 30°C and YGC plates 3 and 5 days at 30°C for yeast and moulds, respectively.

3. Results

3.1. Susceptibility of psychrotrophic food pathogens and spoilage organisms

In Table 2 the amount of time before growth (turbidity) appeared, is summarized for the tested micro-organisms at different chitosan concentrations varying from 40 to 750 mg/l.

The investigated type of chitosan clearly showed antimicrobial activity. Generally, Gram-negative bacteria were more susceptible, while the sensitivity of the Gram-positive bacteria was highly variable: *Brochotrix thermosphacta* and *Bacillus cereus* were very sensitive to the applied chitosan while *Listeria monocytogenes* and different lactic acid bacteria were less susceptible. Yeasts, represented by *Candida lambica* and *Cryptococcus humicolus*, showed an intermediate sensitivity.

3.2. Interaction with food components

The effect of pH on the antimicrobial activity of chitosan on *C. lambica* is illustrated in Fig. 1. The growth of *C. lambica* in Sabouraud medium (Oxoid, CM147) without chitosan was not influenced by the pH. In a medium containing 0.005% (w/v) chitosan the growth was completely inhibited at pH 4.0, while at pH 6.0, the same chitosan concentration led to a significant extension of the lag phase and a rather small decrease in growth rate. These effects were more pronounced as the chitosan concentration was increased to 0.01% ($\lambda = 59.2 \pm 49.0$ h and $\mu_{max} = 0.013 \pm 0.001\ h^{-1}$ in the presence of 0.005% (w/v) chitosan and $\lambda = 216.9 \pm 33.1$ h and $\mu_{max} = 0.011 \pm 0.003\ h^{-1}$ for 0.01% (w/v) chitosan).

Since most foods are mainly composed of water, carbohydrates, fats, proteins and NaCl, it is important to analyse the influence of these components on the antimicrobial activity of any antimicrobial compound.

Starches are used in different forms in the food industry and both the source and the way they were modified can have an influence on their interactions with

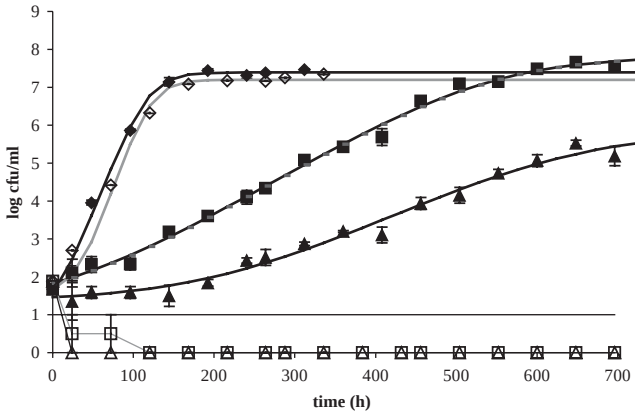

Fig. 1. Influence of medium pH on the effects of chitosan. ♦, without chitosan; ■, 0.005% chitosan and ▲, 0.01% chitosan. Open symbols are pH 4 and closed symbols are pH 6 ($T = 7°C$). The lines represent the fitted Baranyi-model.

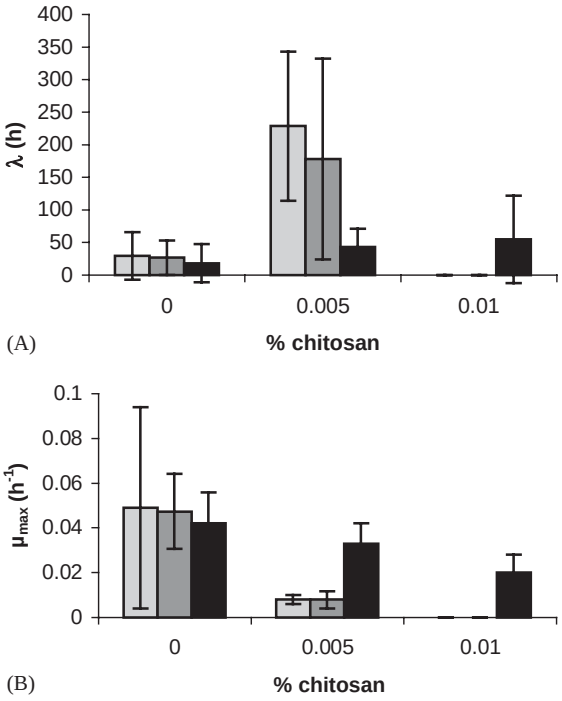

Fig. 2. The influence of soluble starch on the antimicrobial activity of chitosan. Lag phase (A) and growth rate (B) of *C. lambica* with different chitosan and starch concentrations. ($T = 7°C$ and pH = 5). □, 0% starch; ▨, 1% starch; ■, 30% starch

chitosan. In this study a soluble starch was used, which caused a pH decrease in the growth medium. The pH decrease was probably caused by the way of modification (an acid hydrolysis for better solubility) and explains why a readjustment of pH after autoclaving was necessary. Three different chitosan concentrations (0%, 0.005% and 0.01% (w/v)) and starch concentrations (0%, 1% and 30% (w/v)) were tested. Without starch, 0.005% chitosan was enough to cause a significant retardation of the growth of *C. lambica* at pH 5.0 (Fig. 2) and a higher concentration of chitosan even caused inactivation of the yeast. Neither the lag phase nor the growth rate of *C. lambica* was influenced by low amounts of starch (1% (w/v)). On the other hand the activity of chitosan was strongly decreased by high amounts (30% (w/v)) of starch, leading to a significantly shorter lag phase and a significantly higher growth rate (Fig. 2), demonstrating the negative effect of the investigated type of starch on the antimicrobial activity of chitosan. It should be mentioned that only the gelatinized starch was investigated, it is possible that native starch interacts in a different way.

To investigate the effect of proteins on the antimicrobial activity of chitosan, growth experiments with *C. lambica* were performed at two different pH values (4.0 and 6.0) assuring solubility of the whey protein isolates and a different global charge of the protein. At pH 4.0 in the presence of 0.005% (w/v), growth of *C. lambica* was only observed at 10% (w/v) protein concentration (Fig. 3). Increasing the concentration of chitosan to 0.01% (w/v), led to inactivation of the yeast at all the protein concentrations tested. The cell number stayed below the detection limit during the whole incubation period. At pH 6.0 the antimicrobial activity of chitosan was significantly lower than at pH 4.0 (Fig. 1). Furthermore, the activity was completely diminished if 10% whey protein isolate was added. At this protein concentration there were no significant differences anymore between the solutions without chitosan and those with the highest chitosan concentration (0.01% (w/v)) (Fig. 3).

Due to the water activity lowering effect of NaCl, growth retardation of *C. lambica* occurred in samples containing 2% NaCl without chitosan (data not shown). In the presence of chitosan, NaCl (2% (w/v)) had a positive effect on growth as demonstrated by the diminished antimicrobial activity of chitosan (Fig. 4). The influence of NaCl was also visually evaluated in tubes with intermediary NaCl concentrations. It seemed that 1% (w/v) NaCl was already enough to inhibit the antimicrobial activity of chitosan (Table 3).

To determine the influence of fats, a chitosan emulsion was made of chitosan, sunflower oil and Sabouraud medium (Oxoid, CM147). The pH was adjusted to 5.0 since this pH guaranteed a stable emulsion (Jumaa et al., 2002). It seemed that the addition of oil did not influence the growth of *C. lambica* in the emulsion irrespective of the chitosan concentration. A chitosan concentration of 0.01% (w/v) was enough to keep the cell number below the detection limit for the whole incubation period. If 0.005% (w/v) chitosan was added, growth was retarded in relation to the emulsions without chitosan (Fig. 5), but there was no significant difference between the solution with or without fat.

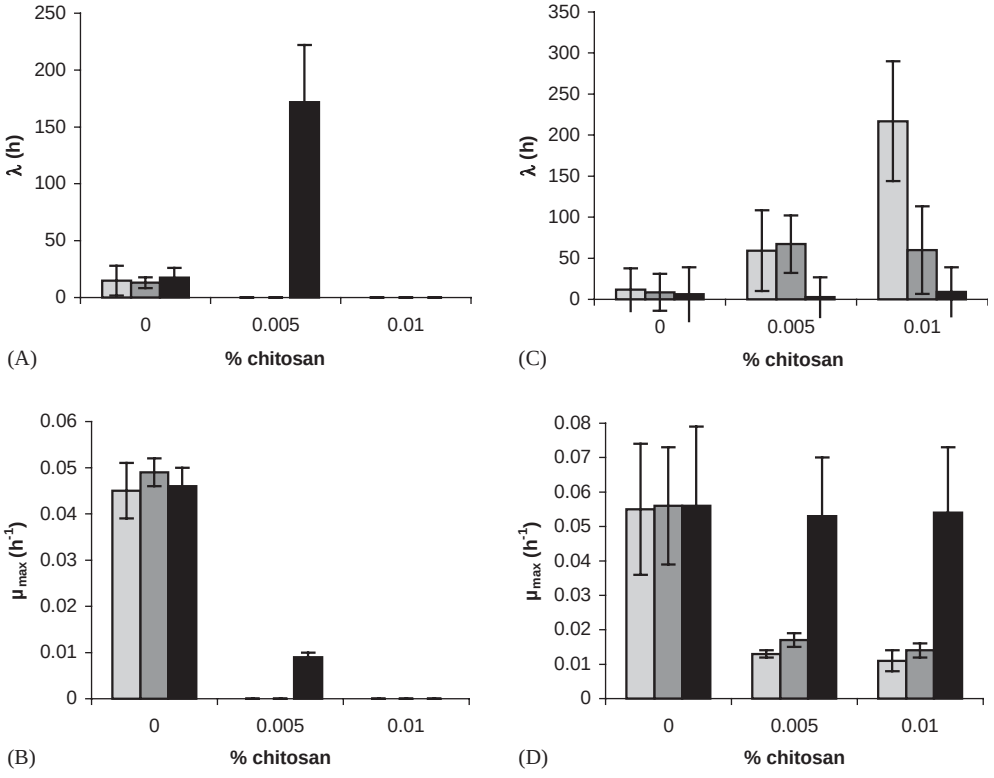

Fig. 3. The influence of proteins on the antimicrobial activity of chitosan at pH 4.0 (A and B) and pH 6.0 (C and D). Lag phase (A and C) and growth rate (B and D) of *C. lambica* with different chitosan and protein concentrations. ($T = 7°C$). □, 0% proteins; ▩, 0.5% proteins; ■, 10% proteins.

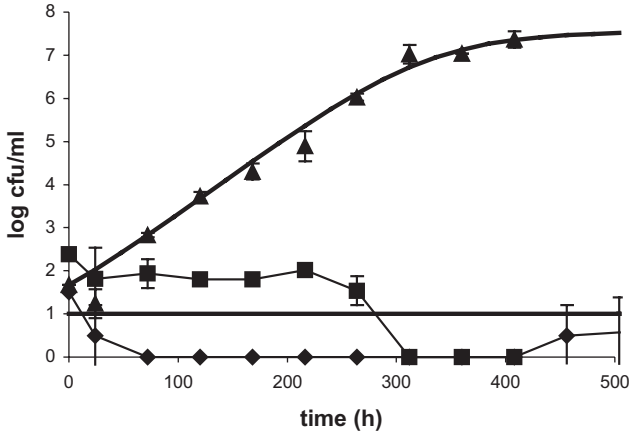

Fig. 4. Growth of *C. lambica* in Sabouraud medium with 0.01% (w/v) chitosan and varying NaCl concentrations: ◆, 0% NaCl; ■, 0.5% NaCl and ▲, 2% NaCl (with fitted Baranyi model) ($T = 7°C$ and pH 5).

Table 3
Growth of *C. lambica* at 0.005% (w/v) chitosan in Sabouraud-medium ($T = 7°C$ and pH 5)

Time (d)	NaCl concentration (% (w/v))				
	0	0.5	1	1.5	2
1	–	–	–	++	++
2	–	–	+	++	++
3	–	–	+	++	++
4	–	–	+	++	++
5	–	–	+	++	++
6	–	–	+	++	++
7	–	–	+	++	++
8	–	–	+	++	++
9	–	–	+	++	++
10	–	–	+	++	++

3.3. In situ experiments

For the in situ experiments the strawberries and mixed lettuce were coated with a chitosan film by dipping them in a chitosan-2% (v/v) lactic acid/Na-lactate solution.

3.3.1. Strawberries

After drying at room temperature, the coating was not visually detectable. Changes in respiration rate, caused by the chitosan film, were measured in order to choose an appropriate package film, with a specific oxygen transmission rate, so that an equilibrium modified atmosphere could be established inside the package. From the experiments it was seen that the respiration rate of strawberries with the chitosan film (12.08 ± 2.04 ml O_2/kg/h) was higher than the untreated samples (5.78 ± 4.32 ml O_2/kg/h) and those treated with a 2% lactic acid/Na-lactate solution only (5.57 ± 3.64 ml O_2/kg/h). All values of respiration rate are calculated at 3% O_2 and 7°C.

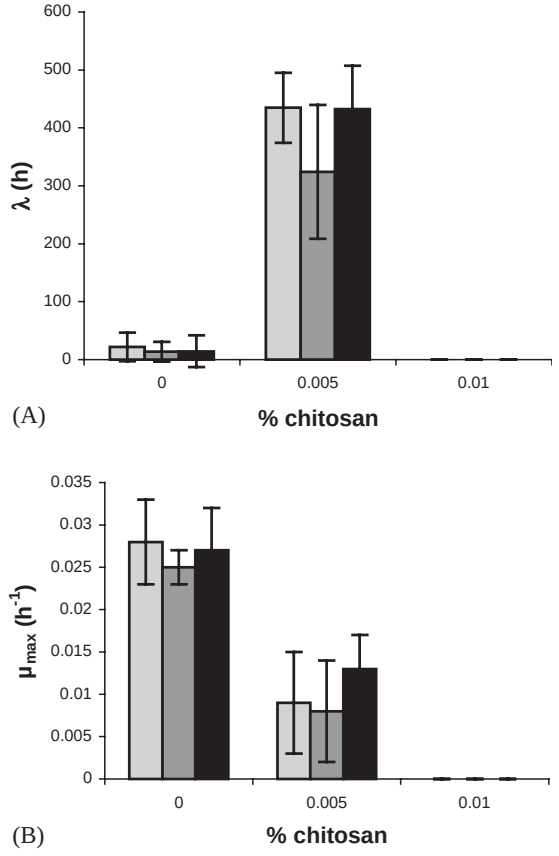

Fig. 5. The influence of oil on the antimicrobial activity of chitosan. Lag phase (A) and growth rate (B) of *Candida lambica* with different chitosan and oil concentrations. ($T = 7°C$ and pH = 5). □, 0% oil; ▩, 1% oil; ■, 10% oil.

3.3.1.1. Analysis of the sensory quality. Sensorial analysis revealed that on the last day of the experiment (day 12) a small odor aberration appeared for all samples while the taste was still acceptable. The samples treated with chitosan were evaluated with a higher score for texture than the untreated samples and those dipped in the lactic acid/Na-lactate solution. Also the juiciness and the color remained optimal during the whole storage period for the three different treatments. On day 0 the strawberries with the chitosan film tasted bitter, but this abnormality disappeared after 3 days of storage at 7°C. Even during further storage, there was no difference between the three treatments on the base of sweetness, sourness and bitterness. The chemical and aberrant tastes were also evaluated, the former was weak to very weak during the whole storage period and the latter was absent for both the untreated and chitosan treated samples.

3.3.1.2. Analysis of the microbiological quality. Mould growth was determined by weighing the strawberries with visible mycelial growth. The results were expressed as a percentage of the original weight of the package. For strawberries the limit had been set on 5% (Hertog et al., 1999; Sanz et al., 1999). From the results (Table 4) it was seen that only on day 12 a significant lower amount of chitosan treated strawberries were rejected in comparison with the untreated samples ($18.97 \pm 9.47\%$ for the treated strawberries and $49.38 \pm 10.47\%$ for the untreated strawberries).

During the storage period the total aerobic psychrotrophic count, total count of lactic acid bacteria and total number of yeasts were measured. The data are summarized in Table 5.

It can be concluded that the chitosan–lactic acid/Na-lactate solution as well as the lactate/Na-lactate solution had an immediately decontaminating activity because on day 0 the microbiological load was significantly lower on the treated samples. The treatments resulted also in a decreased total aerobic psychrotrophic count during the whole storage period. Yeast were the most dominant flora and represented the largest part of the total aerobic count (Table 5).

The number of lactic acid bacteria remained very low during the whole storage period (<3 log cfu/g) from which it can be concluded that lactic acid bacteria seemed not to be important for the deterioration of strawberries. Chitosan was effective in reducing the growth of yeasts on EMAP strawberries; during the whole storage period their numbers were lower when compared to the strawberries treated with lactic acid/Na-lactate alone.

3.3.2. Mixed lettuce

Dipping the lettuce in the lactic acid/Na-lactate solution or chitosan solution resulted in a significantly higher respiration rate (at 3% O_2 and 7°C: 11.93 ± 1.94 ml O_2/kg/h and 13.89 ± 0.72 ml O_2/kg/h, respectively) occurred in comparison with the untreated samples (4.63 ± 1.16 ml O_2/kg/h at 3% O_2 and 7°C).

3.3.2.1. Analysis of the sensory quality. The sensorial evaluation was concentrated on the texture, odor, taste, color and general appearance of the lettuce. Untreated lettuce remained acceptable during the whole storage period (9 days at 7°C) (Fig. 6a) while chitosan treated

Table 4
Weight losses % (w/w) due to mould growth

Day	Treatment		
	Not dipped	0% (w/v) chitosan	0.5% (w/v) chitosan
0	0	0	0
3	0	8.05 ± 11.39	0
5	5.46 ± 7.72	11.52 ± 6.56	17.60 ± 7.81
7	18.81 ± 7.71	12.21 ± 17.27	4.09 ± 5.78
10	20.56 ± 3.52	6.73 ± 0.42	15.36 ± 5.68
12	49.38 ± 10.47	29.98 ± 4.96	17.97 ± 9.47

Table 5
Microbiological analysis on EMAP packed strawberries stored at 7°C (log cfu/ml)

	Day	Untreated	Lactic acid/Na-lactate	Chitosan lactic acid/Na-lactate
Total aerobic psychrotrophic count	0	3.00±0.21	2.00±0.00	2.78±0.25
	3	4.15±0.04	4.02±0.09	2.59±0.16
	7	4.16±0.05	3.15±0.16	3.01±0.01
	10	4.31±0.12	3.76±0.02	3.84±0.82
	12	5.35±0.1	4.12±0.09	3.48±0.11
Lactic acid bacteria	0	1.24±0.34	—	—
	3	2.32±0.30	1.74±0.06	1.39±0.12
	7	2.95±0.67	2.85±1.20	1.39±0.55
	10	2.61±1.85	2.06±0.08	—
	12	1.39±0.12	1.50±0.28	0.65±0.92
Yeast	0	3.07±0.32	2.00±0.00	2.00±0.00
	3	4.08±0.25	4.00±0.00	2.50±0.71
	7	4.58±0.21	4.21±0.27	3.11±0.05
	10	4.05±0.34	3.89±0.04	3.40±0.13
	12	5.11±0.38	4.06±0.03	3.01±0.15

lettuce was unacceptable from day 0. The chitosan–lactic acid/Na-lactate solution was very viscous so that a slimy film was formed on the lettuce surface, which was the major reason for rejection of the samples. On day 0 the lettuce had also a very strong bitter taste but this bitterness disappeared after 2 days of storage at 7°C. After day 4 the lettuce was unacceptable because of the general appearance (Fig. 6b). The cutting surfaces of the lettuce became brown and there were brown spots on several places.

3.3.2.2. Analysis of the microbiological quality. From the total aerobic psychrotrophic count and the yeast count, it can be concluded that there was an immediate decontaminating activity of chitosan (Figs. 7a and b). However, this effect disappeared after 4 days, because at that stage no difference could be noticed between the different treatments. Likewise, the number of lactic acid bacteria remained two log-units lower on the chitosan treated samples. After 9 days there was no difference between the differently treated lettuce (data not shown).

4. Discussion

4.1. Susceptibility of psychrotrophic food pathogens and spoilage organisms

The suitability of chitosan as a food preservative is dependent on the kind of chitosan used and the matrix in which it is dissolved. Therefore, it is important in all experiments with chitosan to use a well-characterized product (polymerization and acetylation degree).

All the experiments in this study were done with chitosan, which had a low polymerization degree (43 kDa) and high deacetylation degree (94%), a combination that seemed to be very favorable for the antimicrobial activity. This can be derived from the relatively low MIC values in comparison with other studies. Generally, Gram-negative bacteria seemed to be very sensitive for the applied chitosan while the sensitivity of the Gram-positive bacteria varied greatly. The MIC for lactic acid bacteria was higher than 0.05% (w/v) while other Gram-positive bacteria were already inhibited at 0.006% (w/v). The MICs reported in the literature for specific target organisms ranged from 0.0018% to 1.0% (Sagoo et al., 2002). Several studies showed *Pseudomonas* sp. to be the most resistant strains (Simpson et al., 1997; Rhoades and Roller, 2000; Jeon et al., 2001). This is inconsistent with our own results and with the studies of Chen et al. (1998), Chen et al. (2002) and No et al. (2002). Some studies demonstrate that yeasts tend to be more sensitive than bacteria (Roller et al., 2002) while our results show an intermediate sensitivity of yeasts for this kind of chitosan (0.01% (w/v)).

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

4.2. Interaction with food components

Apart from the polymerization and deacetylation degree, the antimicrobial activity of chitosan is also very

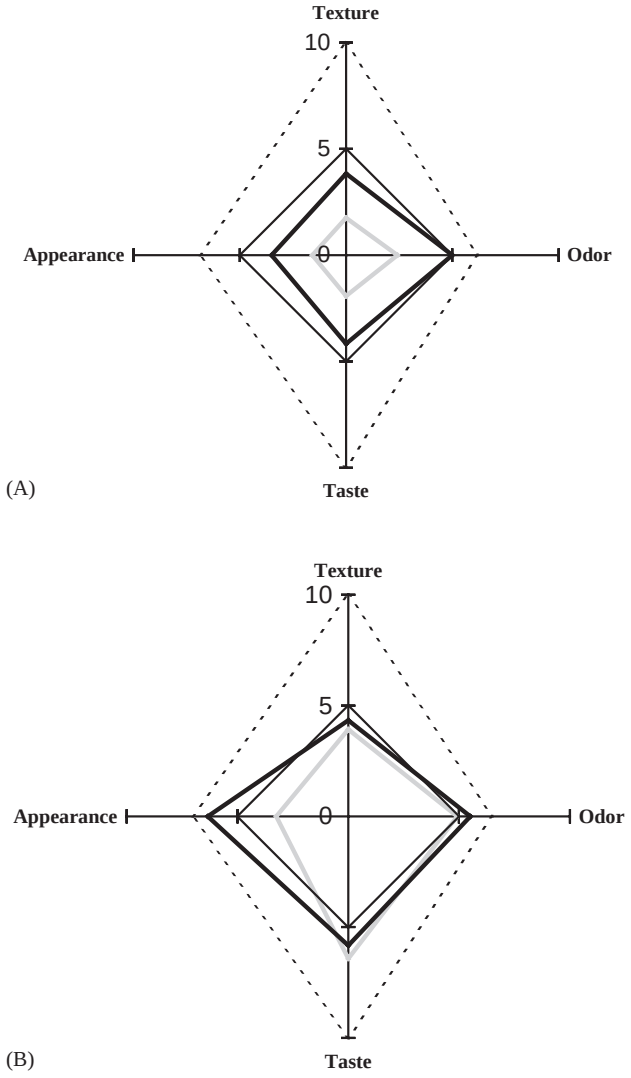

Fig. 6. Evaluation of the general appearance, the texture, the odor and the taste of untreated mixed lettuce (A) and chitosan treated mixed lettuce (B) by a trained panel of 6 persons. Scores were given between 0 (very good) and 10 (very bad). Day 0 (——) day 6 (——) and day 9 (·····) are represented in the figure——, shows the level of acceptance.

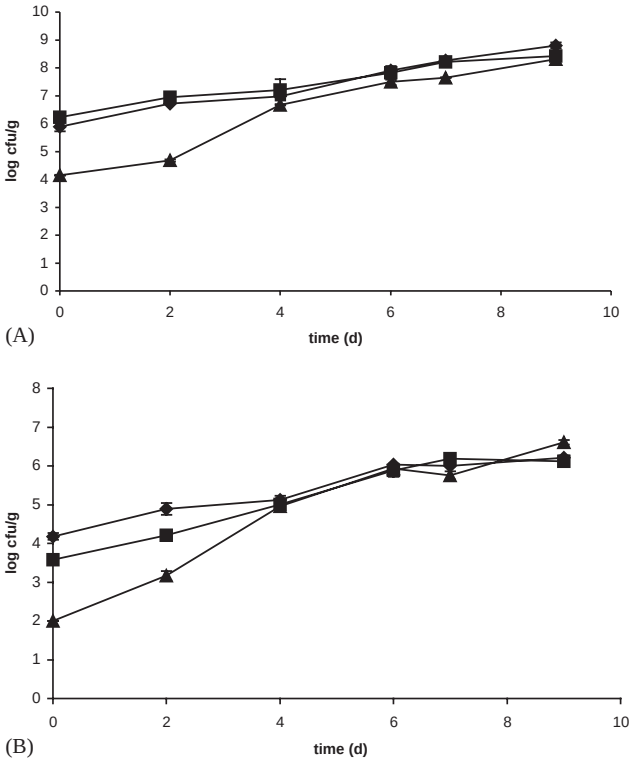

Fig. 7. Evolution of total aerobic psychrotrophic count (A) and yeasts (B) on mixed lettuce during storage at 7°C ♦, untreated lettuce; ■, lactic acid/Na-lactate treated lettuce and ▲, 0.5 (w/v) chitosan-2% lactic acid/Na-lactate treated lettuce.

dependent on the pH of the matrix. The antimicrobial activity is higher at low pH, because more amino groups are protonated (Wicken and Knox, 1983), which leads to (1) a longer persistence length which prevents chitosan from entering bacterial cells (Lyubina et al., 1983) and (2) more interaction with the negatively charged surfaces, which inhibits bacterial growth (Chung et al., 2003). Our study shows that a higher pH leads to a significant reduction of the antimicrobial activity. It can be concluded that a relative small shift in pH can cause a sudden change in the active concentration and can cause a large difference in antimicrobial activity as consequence (Jumaa et al., 2002). Sudarshan et al. (1992) reported that chitosan was no longer bactericidal at pH 7 due to (1) the presence of a significant proportion of uncharged amino groups and (2) the poor solubility of chitosan.

Besides the pH, the antimicrobial activity of chitosan was also very dependent on the matrix. Since the charges on chitosan and the concomitantly electrostatic forces are responsible for the antimicrobial activity, each food component that can influence these interactions will inhibit the activity of chitosan. In our experiments the influence of four different components was investigated separately (starch, proteins, NaCl and fat).

High concentrations of starch (30% (w/v)) inhibited the antimicrobial activity of chitosan. This could be due to a protective effect of starch or to potential electrostatic interactions if the starch would be charged by modification. This result however cannot be generalized for all starches because there can be a great difference between starches with regard to the source and the way of modification. Also lower concentrations of starch (between 5% and 30% (w/v)) must be analysed to make more reliable conclusions.

The influence of proteins is mainly dependent on the pH of the medium, as the charge of the proteins will be determined by the combination of the IEP of the protein itself and the pH of the medium. If the pH is lower than the IEP of the protein then the chitosan will still have good antimicrobial activity. The protein as well as the

chitosan will mainly be positively charged, so the interactions between both will be restricted. If the pH is increased to a value above the IEP of the protein, the antimicrobial activity of chitosan is inhibited. The mainly negatively charged protein will neutralize most of the positive charges on the chitosan in a way that it cannot interact anymore with the negatively charged microbial surfaces. Our results suggest a competition for the positive charges on chitosan between the negative charges on the protein isolate and the negative charges on the cell surfaces of the micro-organisms.

It was seen that the solubility of chitosan improved by adding NaCl to the medium. This can be due to the shielding effect of NaCl against the positive charges, which leads to a coiled structure of chitosan and less interactions with several components in the media (Jiang and Han, 1999). Adding NaCl to the medium will also diminish the antimicrobial activity of chitosan because it interferes with the electrostatic forces between chitosan and the microbial surface. On one hand the Cl^--ions can neutralize the positive charges on the chitosan, while on the other hand the Na^+-ions can compete with chitosan for the negative charges on the cell surface. These observed phenomena are contrary to the observations of Chung et al. (2003). They observed a higher solubility and a higher antimicrobial activity of chitosan with increasing ionic strength.

The influence of fat on the antimicrobial activity of chitosan is negligible and the charges on chitosan seemed to stay available despite the complex formation. This phenomenon could be explained by the fact that chitosan is positioned at the outside of the emulsion drops due to the interaction between the positively charged chitosan and the negatively charged free fatty acids (Jumaa and Müller, 1999). In this way the chitosan formed larger positively charged drops that still maintained their antimicrobial activity. According to the research of Jumaa et al. (2002) there can be a higher antimicrobial activity in the samples with oil. This was ascribed to the immobilization of the microbial cells by hydrophobic interactions between each other and the hydrophobic interactions with the lipophilic molecules.

4.3. Applicability of chitosan coating on fruit and vegetables

Chitosan can theoretically be used as an ideal preservative coating material. It has been shown (1) to inhibit growth of several fungi, except those containing chitosan as wall constituent (Roller and Covill, 1999; Fang et al., 1994; Leuba and Stössel, 1986; Rhoades and Roller, 2000; El Ghaouth et al., 1991a, b, 1992, 2000; Reddy et al., 2000; Jiang and Li, 2001), (2) to induce chitinase, a defense enzyme (Mauch et al., 1984) and (3) to elicit phytoalexinen (Hadwiger and Beckman, 1980; Kendra and Hadwiger, 1984). Due to its ability to form a film, chitosan can be expected to modify the internal atmosphere as well as to reduce the transpiration losses (El-Ghaouth et al., 1991a). Several studies have been done with chitosan as a coating material (Zhang and Quantick, 1998; El-Ghaouth et al., 1991a, b; Li and Yu, 2001). In these studies neither the antimicrobial activity nor the possible taste aberration due to the chitosan film have been evaluated. In our in situ experiments, fruits and vegetables were covered with a chitosan film. The exposure to chitosan increased the respiration of fruits and vegetables, probably because of an induced stress of the lactic acid/Na-lactate solution. This increase in respiration rate slowed down during the storage of the products and, after 4 days, the retarding effect of the chitosan film was noticeable. El-Ghaouth et al. (1991a) observed also an immediate stimulation of the respiration that disappeared gradually. Other fruits showed a decrease in their respiration rate due to the chitosan film over a storage period of more than twenty days (Jiang and Li, 2001; Li and Yu, 2001; El-Ghaouth et al., 1992).

From the taste panel it was seen that chitosan treated strawberries had a better texture, as observed for other fruits, e.g. peaches (Li and Yu, 2001). In some other studies in which the texture of strawberries was measured quantitatively, the firmness of the chitosan-treated samples was significant higher (El-Ghaouth et al., 1991a; Zhang and Quantick, 1998).

For strawberries there where no taste abnormalities perceived for the three different treatments, except on day 0 were the chitosan treated strawberries had a slightly bitter taste. The results from the taste panel on the mixed lettuce, on the other hand, were not satisfactory. On day 0 the products were rejected by the panel because of the bitter taste and the general appearances (slimy), while later they were dismissed because of the induced browning on the cutting surfaces. The introduction of the bitter taste can be due to the higher pH of the dipping chitosan solution in comparison with the one for strawberries or due to the weak taste of the lettuce itself. The microbiological load was for both strawberries and lettuce lower when they were dipped in the chitosan solution. The effect of chitosan disappeared for the lettuce after 4 days. This could be ascribed to the higher pH of the solution leading to a lower antimicrobial activity of chitosan.

5. Conclusions

Before natural preservatives are applied, it is essential to evalute their behavior in food matrices, especially the interaction with food ingredients.

Our experiments illustrate that the effects of chitosan as an antimicrobial preservative for food will be limited to food products with low protein and NaCl content. Fruit and vegetables belong to this category and the

effects of a chitosan–lactic acid/Na-lactate dip solution on the shelf-life of EMAP lettuce and strawberries were therefore tested. A clear antimicrobial activity of the treatment was demonstrated for both products, but its applicability for lettuce will be hampered due to a pronounced bitter taste developed after treatment.

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