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Optimization of chitosan treatments for managing microflora in lettuce seeds without affecting germination

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ABSTRACT

Many studies have focused on seed decontamination but no one has been capable of eliminating all pathogenic bacteria. Two objectives were followed. First, to assess the *in vitro* antimicrobial activity of chitosan against: (a) *Escherichia coli O157:H7*, (b) native microflora of lettuce and (c) native microflora of lettuce seeds. Second, to evaluate the efficiency of chitosan on reducing microflora on lettuce seeds. The overall goal was to find a combination of contact time and chitosan concentration that reduces the microflora of lettuce seeds, without affecting germination. After treatment lettuce seeds presented no detectable microbial counts ($<10^2$ CFU/50 seeds) for all populations. Moreover, chitosan eliminated *E. coli*. Regardless of the reduction in the microbial load, a 90% reduction on germination makes imbibition with chitosan, uneconomical. Subsequent treatments identified the optimal treatment as 10 min contact with a 10 g/L chitosan solution, which maintained the highest germination percentage.

1. Introduction

Minimally processed vegetables, such as lettuce, have recently undergone an increase in consumer demand because of their healthy image and convenience. However, lettuce has been implicated in many foodborne illnesses outbreaks (Beuchat, 1997; Francis, Thomas, & O'Beirne, 1999; Nguyen-the & Carlin, 1994; Solomon, Yaron, & Matthews, 2002; Wachtel, Whitehand, & Mandrell, 2002). The occurrence of foodborne pathogens on fresh lettuce is of particular concern, especially due to bacterial pathogens such as *Escherichia coli* O157:H7, being known by its environmental occurrence (Oliveira et al., 2010; Solomon et al., 2002). It is generally assumed that contamination is due either to handling by infected food handlers, or to preharvest contamination by irrigation water. Postharvest washing treatments may reduce microbial contamination on lettuce, but does not totally remove or inactivate it (Allwood, Malik, Hedberg, & Goyal, 2004). As ingestion of only a few infectious cells may lead to an infection, contaminated salad constitutes a risk to consumers.

Preharvest antimicrobial treatments look for control field infection in order to obtain vegetables with better microbiological quality. Many studies have shown that some bacteria, such as *E. coli*, may become internalized in plant crops (Li, Tajkarimi, & Osburn, 2008; Solomon et al., 2002; Warriner, Spaniolas, Dickinson, Wright, & Waites, 2003). Internalization is of concern because, once inside the plant, the bacteria are protected from postharvest surface washing and sanitizing procedures. In similar studies, the time at which the microorganism was introduced apparently affected the potential for internalization (Warriner et al., 2003). In general, the earlier a pathogen comes in contact with the crop, the greater the potential for a serious disease to develop. This is why it is very important to use clean seeds. Infested seeds can be the source of the pathogen for important bacterial diseases occurring during postharvest management. The first strategy used to control contamination is to eliminate or reduce the amount of pathogen available to initiate the disease. Therefore, the use of disease-free seeds is one of the most important management practices to increase safety.

According to the National Association of State Department of Agriculture (NASDA) seed-borne diseases constitute a demonstrated threat to American Agriculture. Also a testing and/or certification program for seed-borne pathogens is needed to protect agricultural crops. Studies have been done to evaluate the efficacy of different chemical in killing pathogenic bacteria on seeds (Beuchat, 1997; Fett, 2002; Singh, Singh, & Bhunia, 2003). Other seed decontamination methods (like essential oils, ozonized water or aqueous solution of ClO_2) had also been tested in order to reduce microbial counts in different seeds (Beuchat, 1999; Piernas & Guiraud, 1997a, 1997b). Washing seeds with chlorinated water

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(100 mg/L) have reduced natural microflora counts of mung beans by less than 1 log (Splittstoesser, Queale, & Andaloro, 1983). Nevertheless, to date no single treatment has been found completely capable of eliminating all pathogenic bacteria, or one that can be applied to all types of seeds (Scouten & Beauchat, 2002; Singh et al., 2003). Pathogens present in seeds can reach very high populations (>5–6 log CFU/g) from low populations (<1 log CFU/g) during the sprouting process (Stewart, Reineke, Ulaszek, Fu, & Tortorello, 2001) increasing risk do to internalization within the sprout, making more difficult the postharvest decontamination (Itoh et al., 1998).

Washing with water solutions of different sanitizing agents has been tested and some are currently being used. Chlorine is the primary sanitizing agent used; however, it has a negative impact on the environment (Moreira, Ponce, del Valle, & Roura, 2005). A new and demanding market is the organic agriculture. Organic producers are required to use organic seeds. Within this context, there is the need for natural decontamination treatments compatible with organic agriculture. For these reasons, chitosan is presented as a valid option as a natural antimicrobial (Beverlya, Janes, Prinyawiwatkula, & No, 2008; Devlieghere, Vermeulen, & Debevere, 2004).

Chitosan is the second most abundant biopolymer on earth after cellulose and is the linear and partly acetylated (1–4)-2-amino-2-deoxy-β-D-glucan (Muzzarelli, 1977; Ravi Kumar et al., 2004) with many potential applications in the food industry. Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively (Furusaki et al., 1996). It has been found to be nontoxic, biodegradable, biocompatible in addition to having antimicrobial characteristics (Dutta, Tripathi, Mehrotra, & Dutta, 2009). Chitosan is known for having a wide inhibition spectrum for bacteria (Gram-positive and Gram-negative) but also on yeast and molds (Liu et al., 2006). Antimicrobial activity of chitosan is frequently associated to its polycationic structure, being hypothesized to be mediated by the electrostatic forces between the protonated amino group (NH_2) in chitosan and the teicoic acids at cell surfaces (Beverlya et al., 2008). The interaction between the polycationic chitosan and the electronegative charges on the cell surfaces leads to the leakage of intracellular electrolytes and proteinaceous constituents (Chen, Liau, & Tsai, 1998; Devlieghere et al., 2004; Liu, Du, Wang, & Sun, 2004; Muzzarelli et al., 1990). Chitosan antimicrobial actividity is dependant on the strain, the structure of the polymer and the pH of chitosan preparation (Liu et al., 2006; No, Park, Lee, & Meyers, 2002). Moreover, bacteria in different grow stages could have different sensitivity to chitosan (Liu et al., 2006).

Since the use of chitosan solutions as a seed disinfectant is relatively new, not much information is available about its antimicrobial growth. Appropriate applications need to be validated, and a novel approach for the development of effective, ecologically friendly and cheap seed decontamination technology is still needed (Luksiene et al., 2007). To obtain the highest sanitizing agent performance it is very important to optimize the contact time between the target microorganism and chitosan solutions, as well as the concentration of chitosan to be used.

Two initial objectives were followed in the present work. First, the assessment of the *in vitro* antimicrobial activity of a commercial chitosan against three microbial populations: (a) *E. coli O157:H7*, (b) native microflora of fresh Butterhead lettuce and (c) native microflora of Butterhead lettuce seeds. The second objective was to evaluate the effectiveness of chitosan treatments for reducing microflora on lettuce seeds. However, the overall goal of the work was to find the appropriate combination of contact time and chitosan concentration that significantly reduces the microflora of Butterhead lettuce seeds, without affecting the germination percentage.

2. Materials and methods

2.1. Biopreservative solutions preparation

Chitosan solutions (5, 10 and 20 g/L) were prepared by dissolving chitosan powder (ACOFAR, Mar del Plata, Argentina; 98% deacetylation degree) in acetic acid solution 1% (v/v) (Moreira, Pereda, Marcovich, & Roura, 2011), pH was adjusted to 5.6 and mixed overnight at 100 rpm in an orbital shaker (TS-1000, Zhejiang, China).

2.2. Inoculum preparation

Fresh Butterhead lettuce (*Lactuca sativa* var. *Lores*) was obtained from a local producer in the vicinity of Mar del Plata, in the southeast of Buenos Aires Province, Argentina. Lettuce leaves with evident physiological damage were discarded. Ten grams of the remaining leaves were macerated in 90 mL of phosphate-buffered solution (pH = 7.2) according to Ponce, Roura, del Valle, and Fritz (2002) in a tissue homogenizer (Stomacher 400 Circulator Homogenizer). The homogenate obtained was statically incubated overnight at 37 °C. Lettuce seed inoculum was equally obtained.

E. coli O157:H7, ATCC 25158 (American Type Culture Collection) was used. Stock culture was maintained on Tryptic Soy Broth (TSB, Britania) at 4 °C. Before it was used, the *E. coli* was cultured in Brain Hearth Infusion broth (BHI, Britania) for 24 h at 37 °C. Approximately 0.1 mL of culture was transferred to 9.0 mL of BHI at 2 consecutive 24 h intervals immediately before each experiment

2.3. In vitro assay

Test tubes with 6 mL of brain heart infusion (BHI) broth were inoculated with 1 mL of bacterial inoculums (approximately 10^7 colony-forming units, CFU/mL). After that, 3 mL of chitosan solution at different concentrations (5, 10, 20 g/L) was added and followed by shaking for 30 s at 1800 rpm (Moreira et al., 2011). The system was statically incubated at 35 °C for 48 h. Microbial evolution was followed by optical density (OD) determination using a spectrophotometer (SP-2000UV Spectrum, Zhejiang, China) at 610 nm. A 2 mL aliquot of each tube was measured at 0, 3, 6, 24 and 30 h to determine the inhibition of the target microorganisms (native Butterhead lettuce, native lettuce seed and *E. coli*). Control tubes without chitosan were tested in the same way. Two independent runs were performed to analyze the effect of chitosan on the microflora of lettuce, seeds and *E. coli*; and the assays were done by triplicate.

2.4. Imbibition assay

Imbibition was defined as the time, in minutes, needed for the seed reach the stationary phase, where respiration starts and the imbibition rate slows down (Bewley, 1997). In the present study, imbibition was carried out following the methodology proposed by Wierzbicka and Obidzinska (1998) with modifications. 0.5 g of lettuce seeds were weighed (0.001 g precision) and then immersed in 30 mL of different imbibition solutions: distillated water, and chitosan (5, 10 and 20 g/L) in 1% (v/v) acetic acid. Afterward the different solution-seed systems were stirred in an orbital shaker (1000 ST, Zhejiang, China) at 100 rpm. Every 15 or 30 min seeds were dried and weighed.

Weight seed rate during imbibition is measured as the amount of water taken up (Bewley, 1997). The amount of imbibed water at each sampling time was calculated following Eq. (1), where w_t and w_i represent the weight of the lettuce seeds (g) at each sampling

time and the initial weight, respectively; while δ_{H_2O} corresponds to water density (1 g/mL).

$$\text{IW (mL)} = \frac{(w_t - w_i)}{\delta H_2O} \quad (1)$$

Imbibition was carried out by triplicate and three independent runs were performed. Results are presented as lsmean (least square mean) values with the corresponding standard error.

Imbibition time for all different treatment solutions was determined as a function of the total imbibition water that seeds incorporate until reaching the stationary phase. The total water volume (mL) was calculated as the mean value resulting of two consecutive measurements that showed no significant differences for each treatment. Afterward, contact time was defined as the time needed (min) for lettuce seeds to incorporate 75% of the total imbibition water volume.

2.5. Germination assay

Immediately after seed imbibition with the different chitosan solutions and control treatments, 50 lettuce seeds were sown in plastic trays (18 cm × 14 cm) with two layers of Whatman filter paper #42, adequately moistened with 20 mL of distillated water. Then trays were covered with plastic foil to prevent dehydration and incubated in a germination chamber (20–22 °C and 8 h photoperiod) for 7 days. Percentage of seed germination was determined according to Barassi, Ayrault, Creus, Sueldo, and Sobrero (2006) by counting germinated seeds at day 7. Only those seedlings without defects were considered as germinated. In order to determine the absence of defects, each tray was thoroughly inspected with a magnifying glass while germinated seedlings were counted. Each treatment was performed by duplicate and three independent runs were carried out.

To find the optimum contact time, the germination percentage was also determined after different imbibition times (5, 10, 15 and 20 min) with the different treatment solutions.

2.6. Microbiological studies

Microbial population counts were performed with the following culture media and incubation conditions: mesophilic aerobic bacteria on plate count agar (PCA) incubated at 37 ± 1 °C for 48 ± 3 h, molds and yeast were counted in yeast–glucose–chloranphenicol (YGC) medium at 25 °C for 5 days and *Enterobacteriaceae* and total coliforms in bile crystal violet neutral red (MacConkey) agar with the addition of superficial virgin layer incubated at 30–32 °C for 24 h (ICMSF, 1983). Presumptive *E. coli* colonies from MacConkey agar were spread on the surface of eosin methylene blue (EMB) agar, as a selective culture medium for Gram-negative bacteria and incubated at 37 °C ± 1 °C for 24 ± 2 h (Leininger, Roberson, & Elvinger, 2001).

The effect of the chitosan treatment over the seed microbial load was analyzed immediately after imbibition. Samples of lettuce seeds were subjected to imbibition treatments, dried up in sterile towel paper. For each treatment, a sample of 50 seeds was homogenized in 10 mL of phosphate-buffered solution (pH = 7.2) and vigorously mixed by 60 s. Aliquots of each supernatant were used for microbial determinations as previously described, by duplicate. Results are presented as the mean of three independent replicates, expressed as $\log_{10}$ CFU/50 seeds where the lower detection limit of this technique was 2 $\log_{10}$ CFU/50 seeds.

2.7. Statistical analysis

Data were analyzed using SAS, software version 8.0 (SAS Inc., 1999). PROC GLM (general linear model procedure) was used for the analysis of variance (ANOVA). PROC UNIVARIATE was used to validate ANOVA assumptions. Results reported in this paper are lsmean values (least square mean, means estimators by the method of least squares) together with their standard error (Kuehl, 2001). The factors employed as sources of variation were TIME (imbibition or incubation time, according to imbibition or microbiological assays, respectively) and TREATMENT (different % (w/v) chitosan solutions), and TIME–TREATMENT interaction. When significant differences were found with the GLM procedure, the Tukey–Kramer multiple comparison test was carried out ($p < 0.05$).

For germination assays, where results correspond to proportions instead of normally distributed data, PROC FREC was more appropriate to be used. Chi-square test was used to find out if the proportions were dependent of the treatment or not ($p < 0.05$). In these assays, PROC RANK was used to group the proportions in order to select the treatment with higher germination rate.

Table 1
p-Values of the ANOVA (by treatment) for control and chitosan solutions against the INDICATOR microorganisms (lettuce: native microflora of Butterhead lettuce, seed: native microflora of Butterhead lettuce seeds and *E. coli* O157:H7) from OD values, previously presented in Fig. 1.

Treatment	Indicator microorganisms		
	E. coli	Lettuce	Seeds
Control	0.0034	0.0028	0.0020
Chitosan 5 g/L	0.0016	0.0228	0.0172
Chitosan 10 g/L	***0.0874***	***0.5021***	***0.0844***
Chitosan 20 g/L	***0.1490***	***0.3665***	***0.0969***

Bold and italic values highlighted indicate treatments without significant increment of OD ($p > 0.05$)

3. Results and discussion

3.1. In vitro assay

Optical density (OD) determination is a reliable technic to quantify the biopreservative action of any treatment on the evolution of different microorganisms. This is especially adequate for chitosan solutions, for which the Agar Diffusion Methods tends to produce false negative results, possibly due to the high molecular weight of the polymer. Moreira et al. (2011) reported not significant antimicrobial effects of chitosan on the native microflora of carrots. Chitosan high viscosity limits diffusion into the agar medium causing the seemingly low antimicrobial activity (Moreira et al., 2011). Similar results were described in several studies when agar diffusion method is used to assess the antimicrobial effect of chitosan (Coma, Martial-Gros, Garreau, Copinet, & Deschamps, 2002; Ponce, Roura, del Valle, & Moreira, 2008; Zivanovic, Chi, & Draughon, 2005).

Fig. 1 shows the OD evolution obtained for the studied microorganisms in the presence of the different chitosan solutions. The statistical analysis of the experimental data showed a significant interaction TREATMENT–TIME ($p < 0.0001$) for all the analyzed microorganisms. Those treatments without significant increment of DO were considered effective to inhibit the development of the indicator microorganisms. The corresponding *p*-values for the control and chitosan solutions are presented in Table 1.

Chitosan solutions at 10 and 20 g/L resulted in a significant reduction of the three indicator microorganism's growth. Increments in OD (8–12 folds) were observed in control samples respect chitosan solutions. Similar results were reported by Moreira et al. (2011) testing chitosan, mainly against yeasts and molds.

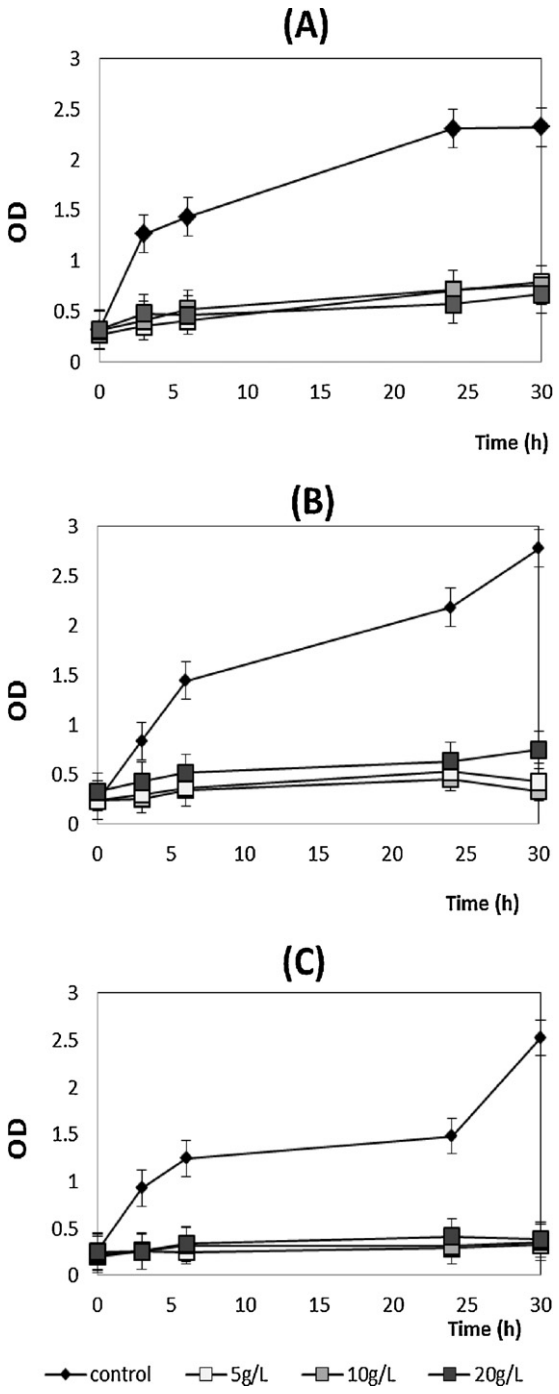

Fig. 1. Optical density (OD) obtained for the different indicator microorganisms: (A) *E. coli O157:H7*, (B) native lettuce microflora and (C) native microflora from lettuce seeds. Values reported as lsmeans (least square means, means estimators by the method of least squares) and vertical bars corresponding to standard deviation.

3.2. Imbibition assay

Fig. 2 shows the profiles obtained for lettuce seed imbibitions using different chitosan solutions. The imbibition process presented two distinctive stages: a rapid imbibition and a stationary one.

ANOVA was performed using TREATMENT, TIME and TREATMENT–TIME interaction as variation source. A non-significant interaction was found ($p = 0.0634$) while both main effects were significant ($p = 0.006$ for treatment and $p < 0.0001$ for time). Table 2 presents the lsmeans values of the amount of water

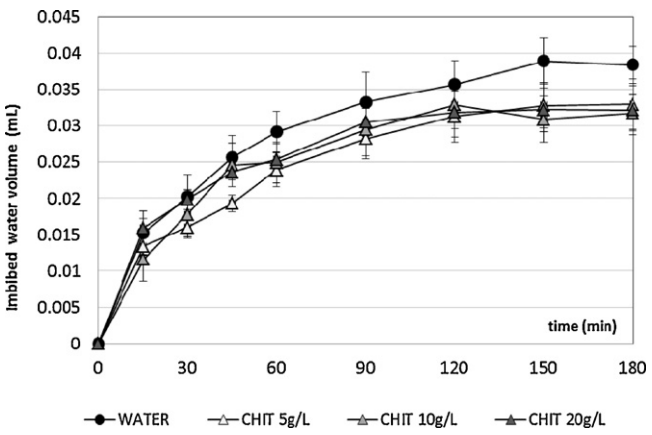

Fig. 2. Imbibition curve of Butterhead lettuce seeds. Values reported as lsmeans (least square means, means estimators by the method of least squares) and vertical bars corresponding to standard deviation.

(mL) imbibed, at each sampling time for all treatments. When at least two consecutive values were none significantly different ($p > 0.05$) according to Tukey–Kramer multiple comparison test the imbibition stationary stage was established. For all treatments, this stage was reached after 90 min. No significant differences in the maximum volume of imbibed water were obtained for the treatments, with a mean value of 0.0335 mL. Barassi et al. (2006) also reported similar results working with lettuce seed.

In the present study, imbibition was proposed in order to facilitate the biopreservative penetration into the seed, pointing toward eliminate not only superficial microflora but also any internalized one. From data shown in Table 2, it was determined that the time needed to reach the 75% of the total volume of water (0.025 mL) entering the seed was 60 min for all treatments. As a consequence, the following germination and microbiological studies were carried out with a 60 min contact time.

Additional statistical analysis of the interaction was carried out, but using TIME as source of variation. Not significant differences ($p = 0.5862$) were found in the imbibed water volume after 60 min of process, with a mean value of 0.026 mL. This indicated that the presence of the chitosan in the imbibition solution did not affect the imbibition rate compared to water control.

3.3. Imbibition effect on microbial counts

Total mesophylic and total yeast and molds counts, before lettuce seed imbibition, were about 5 log CFU/50 seeds. High coliform counts were found in the lettuce seeds (3.46 log CFU/50 seeds). Presumptive colonies of *E. coli* (red colonies with a turbid precipitated halo) were found using a selective culture medium (McConkey). These colonies were transferred to EMB agar, in which *E. coli* was presented as typical colonies with metallic green sheen with dark center). Several studies carried out on different seeds and sprouts also detected high coliform counts. Prokopowich and Blank (1991) reported a microbiological analysis of commercial sprouts, indicating aerobic plate counts and coliforms ranging from 30×10^2 CFU/g to 40×10^5 CFU/g and from 0 to 11×10^3 CFU/g, respectively. Piernas and Guiraud (1997a) reported that microflora on rice seed exceed 10^7 CFU/g. Potter and Ehrenfeld (1998) detected non-O157 *E. coli* in 5 of 48 samples of mung bean seeds indicating possible fecal contamination. Alfalfa and bean sprouts, among which many microbiological studies had been performed over the years, have been shown to contain 10^8 to 10^9 CFU/g (Patterson & Woodburn, 1980) and 6 of 23 alfalfa retail samples contained more than 10^5 fecal coliforms/g. However, lettuce seeds had not

Table 2
Water volume (mL) imbibed in lettuce seed treated with different % (w/v) chitosan solutions and water.

Time (min)	Water volume imbibed (mL)			
	Water	Chit 5 g/L	Chit 10 g/L	Chit 20 g/L
0	0.0000[a]	0.0000[a]	0.0000[a]	0.0000[a]
15	0.0141[b]	0.0122[b]	0.0104[b]	0.0170[b]
30	0.0218[c]	0.0175[c]	0.0154[bc]	0.0217[bc]
45	0.0266[c]	0.0179[c]	0.0216[c]	0.0222[c]
60	0.0285[c]	0.0255[d]	0.0269[c]	0.0264[cd]
90	0.0371[d]	0.0263[de]	0.0308[cd]	0.0323[d]
120	0.0389[d]	0.0328[e]	0.0305[d]	0.0296[d]
150	0.0330[d]	0.0337[e]	0.0324[d]	0.0305[d]
180	0.0355[d]	0.0306[e]	0.0300[d]	0.0334[d]
Time needed to reach maximum volume (min)	90	90	90	90
Maximum volume (mL)[a]	0.0361	0.0323	0.0301	0.0308
75% of max. Volume (mL)	0.0271	0.0242	0.0226	0.0231
Time needed to reach 75% of maximum volume (MIN)	60	60	60	60

Means in the same row followed by different superscripts are significantly different as determined by Tukey–Kramer multiple comparison test ($p < 0.05$).
[a] Calculated as the mean value after the imbibition stationary stage was reached.

been subject of many studies on the naturally occurrence microbial population.

In the present study, imbibed lettuce seeds with chitosan solutions (5, 10 and 20 g/L) presented microbial counts significantly lower ($p < 0.001$) than seeds without treatment or seeds imbibed in water (Table 3). Potter and Ehrenfeld (1998) reported that simple water washing of bean sprouts was not effective in the complete reduction of *E. coli* and *Salmonella* (no more than 1 log). After imbibition, total mesophilic bacteria, molds, yeast and total coliforms were not detectable (<10^2 CFU/50 seeds) in all seeds treated with chitosan solutions. Other studies, using sanitizing agents no compatible with organic seeds, reported microbial counts reductions in seeds and sprouts. Piernas and Guiraud (1997b) observed 2–3 log reductions in aerobic plate counts from rice seeds after treatment with 1000 ppm NaOCl or 10,000 ppm H_2O_2 at room temperature. Washing mung bean seeds with water containing 100 ppm chlorine reduced the microflora by less than 1 log (Splittstoesser et al., 1983), and treating mature sprouts with 5000 ppm chlorine resulted in a 2 log reduction (Moline & Kulik, 1997).

In this context, chitosan as a natural sanitizing agent could act as effective intervention agents to reduce external surface microflora of lettuce seeds. Chitosan solution proved to reduced approximately 5 log the microbial population counts of lettuce seeds (total mesophilic, yeasts and molds and coliforms). Moreover, chitosan eliminated *E. coli* since it was present in the untreated and water imbibed seeds while it was not detected in chitosan treated seeds.

3.4. Germination assay

For germination assays, lettuce seeds were imbibed for 60 min and then placed in a germination trays soaked with distillated water. After 7 days in the germination chamber the proportion of germinated seeds in each tray was calculated. Results are shown in Fig. 3.

Table 3
Microbial counts in lettuce seeds (TM: total mesophyllic bacteria, TYM: total yeasts and molds, TC: total coliforms) immediately after treatment (60 min at 100 rpm).

	TM	TMY	TC
CONTROL	5.06 ± 0.74	5.14 ± 0.71	3.46 ± 0.62
WATER	4.07 ± 0.86	4.71 ± 0.59	2.74 ± 0.74
CHITOSAN	<2	<2	<2

CHITOSAN: chitosan solutions (5, 10 and 20 g/L); CONTROL: without treatment. Values reported are lsmeans (least square means, means estimators by the method of least squares) ± standard deviation.

A clear inhibitory effect was observed for all chitosan treatments compared to untreated seeds or those imbibed with distilled water. Acetic acid was used as diluent for chitosan, because pH lower than 6 is required for its appropriate dissolution (Fernandes, Tavaria, Soares, Ramos, & Monteiro, 2008) and to maintain its polycationic structure, believed to be responsible for the antimicrobial activity (Devlieghere et al., 2004). The acidity of the imbibition solutions (pH = 5.4) might be responsible for the perceived reduction in the germination rate. Germination is a complex process, tightly regulated by hormones and biochemical processes (Matilla & Matilla-Vázquez, 2008) and dependent on the active transport of substances through the seed coat. Given that many biochemical processes are regulated by enzymes and it is well known that enzymatic activity is strongly affected by pH, the inhibition of germination might be expected in the presence of chitosan solutions. Lynch (1980) also found that acetic acid applied exogenously inhibited root extension in barley seeds.

Effective seed decontamination treatments must inactivate microbial pathogens, while preserving seed viability, germination and vigor (Luksiene et al., 2007). In the present study, the 90% reduction on the germination percentage obtained in treated seeds, regardless of the significant reduction in the microbial load (Table 3), makes the proposed treatments highly uneconomical. It would be impossible for any of these treatments to be implemented by farmers; this should always be considered when new technology is developed.

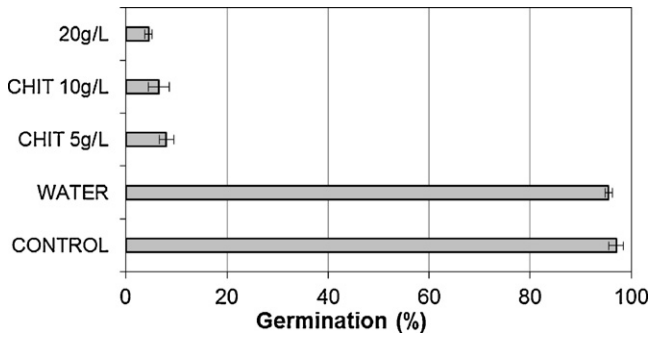

Fig. 3. Germination percentage of lettuce seeds after imbibition with different biopreservative solution concentrations (chit: chitosan) during 60 min at 100 rpm. Values are lsmeans (least square means, means estimators by the method of least squares) and horizontal bars represent standard error.

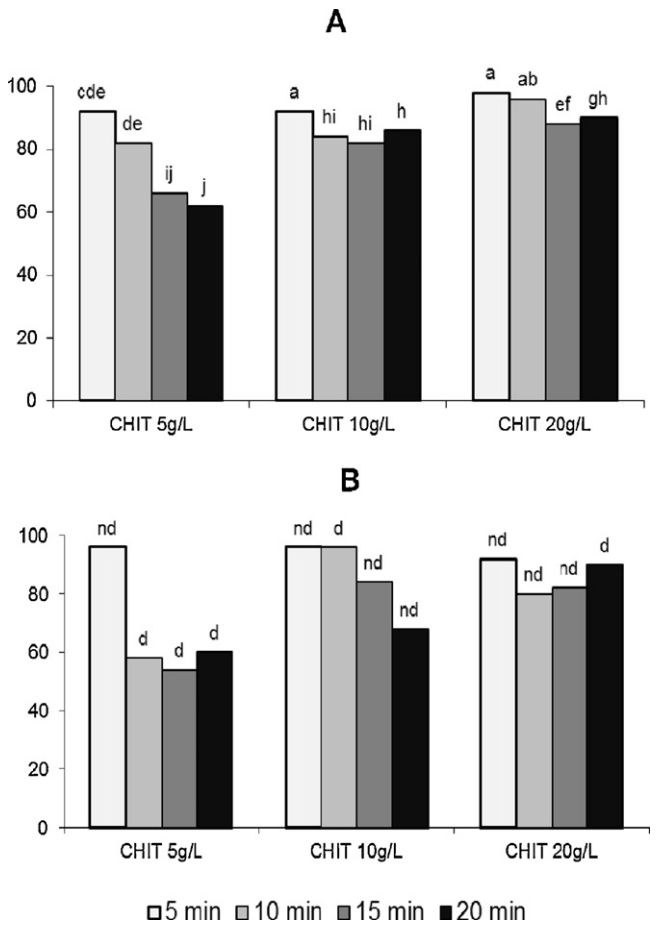

Fig. 4. Germination percentage of lettuce seeds (at day 7) after different imbibition times (5, 10, 15 and 20 min) and different % (w/v) chitosan solutions (5, 10 and 20 g/L). (A) Without subsequent rinsing with distilled water and (B) with subsequent rinsing with distilled water (10 min at 100 rpm). In (A) different letters indicate significant differences by LSD test ($\alpha = 0.05$). In (B) nd (nondependent) and d (dependent) indicates if germination percentage is affected or not by rinsing next to imbibition, according to Chi-square test ($p < 0.05$). Values reported as lsmeans (least square means, means estimators by the method of least squares).

Shorter imbibition times were tested against different chitosan solution with the objective of finding a suitable combination of time–concentration that allows germination whilst reduces microbial populations present in the seed. The reduction on the contact time may limit the detriment of the enzymatic activity and the amount of damage on the cell coat in the seeds. Results of imbibition with shorter time, with and without a subsequent rinse with distillated water, are presented in Fig. 4A and B, respectively.

An association test among proportions (Chi-square test) was performed to determine if rinsing the seeds after imbibition had an effect on the germination percentage of lettuce seeds. Only in 6 out of 16 treatments there was an association between these two events (Fig. 4B). As a conclusion, it can be assumed that rinsing the seeds after imbibition had no positive effect on the germination percentage. Moreover, as the aim in the present study is to propose a useful method to reduce microbial counts in lettuce seeds that can be easily implemented by producers, rinsing the seeds after imbibition should not be considered since the extra activity did not produce any significant improvement.

Selection of the most suitable contact time–concentration combination was focused on finding those treatments with similar germination percentage to the control seeds (98%). The group with higher germination percentage (96%) was comprised with three treatments: 5 min with 5 g/L of chitosan and 5 min and 10 min with 10 g/L of chitosan. Increasing chitosan concentration to 20 g/L produced a decreased in germination (10–20%) for all contact times. In the same way, increasing contact time to 15 or 20 min, significantly reduce germination (20–60%). As no significant differences were found among the combination of treatments (5 min, 5 g/L; 5 or 10 min, 10 g/L) 10 g/L chitosan was selected as the one with more biopreservative concentration that did not reduce germination; and 10 min was chosen since it was the longer period during which seeds can be subjected to imbibition without affecting its germination.

4. Conclusion

In the present study, a suitable protocol for lettuce seed decontamination using a natural biopreservative compatible with organic seed disinfection was established. Imbibition of lettuce seeds with different % (w/v) chitosan solutions proved to be highly effective in eliminating the superficial microflora, including *E. coli*. Chitosan solutions (10 g/L) significantly reduce the mesophilic bacteria, the coliforms and the yeast and molds that naturally appear in the seeds after a shorter period of imbibition (10 min) without affecting significantly its germination percentage. The proposed protocol consists of immerse the seeds for 10 min in the decontamination solution (10 g/L chitosan with 1% (w/v) acetic acid as diluent) to be germinated later.

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