J Food Sci Technol
DOI 10.1007/s13197-016-2484-5

ORIGINAL ARTICLE

Lactic acid as potential substitute of acetic acid for dissolution of chitosan: preharvest application to Butterhead lettuce

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Revised: 26 December 2016 / Accepted: 30 December 2016

Abstract Chitosan must be dissolved in acid solution to activate its antimicrobial properties. The objectives of present study were to determine whether acetic and lactic acids used to dissolve chitosan would influence its effectiveness to control the native microflora of Butterhead lettuce at harvest and during postharvest storage (7–8 °C, 5 days). Chitosan was applied as a SINGLE DOSE (14, 10, 7, 3 or 0 days previous to harvest) or in SUCCESSIVE DOSES (at 14 + 10 + 7+3 + 0 days prior to harvest). Although chitosan in acetic acid showed antimicrobial activity, treated plants showed dried brown stains which significantly reduced sensorial quality. Chitosan in lactic acid applied in a SINGLE DOSE at harvest or in SUCCESSIVE DOSES reduced microbial counts of all populations at harvest without affecting sensorial quality. After postharvest storage, lettuce treated with SUCCESSIVE APPLICATIONS of chitosan in lactic acid presented significant reductions in the microbial populations compared with untreated sample (−2.02 log in yeast and molds, −1.83 log in total coliforms, −1.4 log CFU g^{-1} in mesophilic bacteria and −1.1 log in psychrophilic bacteria). In conclusion, replacement of acetic by lactic acid did not affect the antimicrobial activity of chitosan, reducing microbial counts at harvest and after postharvest storage without affecting sensorial quality.

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Keywords Biopreservative · Safety · Greenhouse · Organic · Sensorial quality

Introduction

Along with the increase in lettuce consumption, a rise in the number of outbreaks of illness associated with raw or minimally processed lettuce has also occurred (Huang and Chen 2011; Kim et al. 2006; Lee et al. 2002). Recent changes in dietary habits, production methods and processing, sources of produce, and the emergence of pathogens previously not recognized for their association with raw produce have enhanced the potential for outbreaks of foodborne illness (Beuchat 2002). Microbiological contamination originates from irrigation water, animals, pests, fertilizers, infected workers, and food processing facilities with poor sanitation (Lee et al. 2004).

Lettuce can be contaminated with microorganisms, both spoilage and pathogenic, while growing, or during harvesting, postharvest handling, processing or distribution. Although spoilage bacteria, yeasts and molds dominate the microflora of raw fruits and vegetables, the occasional presence of pathogenic bacteria, parasites and viruses capable of causing human infections has also been documented (Beuchat 2002). Therefore, treatment with sanitizers is a very important step for preventing these incidents of foodborne outbreaks (Choi et al. 2012) and increasing shelf-life of the final product.

Most consumers assumed that washing and sanitizing reduce the microbial load present in lettuce heads. Although current techniques used by the fresh vegetable processing industry have improved the overall quality and extended the shelf-life of these products, but safety is still a concern (Olaimat and Holley 2012). Among

Published online: 13 February 2017

fresh vegetables, Butterhead lettuce presents a particular challenge since the leaf arrangement in the head (concentric rings around the stem) favors the internalization of microorganisms, protecting them against subsequent disinfection procedures (Goñi et al. 2010).

Today, there is a worldwide trend to explore new alternatives against chemical treatments that control microbial growth, giving priority to methods that avoid negative side effects on human health or the environment (Biji et al. 2015; Gol et al. 2015; Meng et al. 2008). In addition to this latter issue, the appearance of pathogens resistant to the synthetic fungicides is a growing issue of concern (Badawy and Rabea 2009; Edirisinghe et al. 2014).

Chitosan has become a promising alternative treatment for fruit and vegetables due to its non-toxicity, antimicrobial activity, and elicitation of defense responses in plant tissue (Biji et al. 2015; Edirisinghe et al. 2014; Mohammadi et al. 2015). Moreover, as a non-toxic biodegradable polymer, as well as an elicitor, chitosan has the potential to become a new class of plant protectant, assisting towards the goal of sustainable agriculture (Bautista-Baños et al. 2006). Some research has been done in its preharvest application on horticultural crops, both as an elicitor and as an antimicrobial or antifungal agent (Badawy and Rabea 2009; Meng et al. 2008; Romanazzi et al. 2009). However, little information is available for its preharvest application in lettuce.

Chitosan has been proven to control numerous pre and postharvest diseases on various horticultural commodities. It has been reported that both soil and foliar plant pathogens (fungal, bacterial and viral) may be controlled by chitosan application (Bautista-Baños et al. 2006). Postharvest application of chitosan combined with *Zataria multiflora* or *Cinnamonum zeylanicum* essential oils inhibited *Botritis cinerea* rot in strawberries after 9 days of storage (Mohammadi et al. 2015). It was also used as an antimicrobial agent in pasteurized palm sap (*Borassus flabellifer* Linn.) where a reduction of microbial counts was found when compared to control (Naknean et al. 2015). Although the agriculture use of chitosan to control plant pathogens has been extensively explored with more or less success depending on the phyto-system, scarce studies have reported the bactericidal or bacteriostatic effects of chitosan applied as a preharvest intervention step to improve the safety of fresh produce. *In vitro* studies or postharvest application of chitosan solutions or edible films are promising in the effectiveness of chitosan in controlling microbial proliferation on vegetables (Biji et al. 2015; Gol et al. 2015). However, generalization is not possible and optimization of the application of chitosan in metabolic active tissue (like growing lettuce) is needed.

Antimicrobial activity of chitosan has been previously demonstrated in vitro (Goñi et al. 2013a). However, such results can hardly be extrapolated to complex food systems, as a living plant, because interaction with the tissue constituents may impaired its actions, besides other important changes related to sensorial properties of the food matrix (Fernandes et al. 2008). Chitosan is soluble in diluted acidic solutions, below pH 6.0 because the presence of primary amino groups with a pKa = 6.3 and requires an acidic environment to form a steady solution (Rinaudo et al. 1999). At low pH, the amines get protonated and become positively charged making chitosan a water-soluble cationic polyelectrolyte. As the pH increases, chitosan amines become deprotonated and the polymer loses its charge and becomes insoluble (Romanazzi et al. 2009).

Early intervention has the advantage of reducing microbial populations of fresh cut vegetables, preventing economical loses and reducing the ever present risk of illnesses associated to the consumption of raw vegetables, especially leafy vegetables which are very difficult to sanitize (Lee et al. 2004). Preharvest application of chitosan has been mainly focused on the stimulation of plant defense, by triggering a defense response within the plant or the formation of physical and chemical barriers against invading pathogens. However, it may also have a significant impact on the native microflora, reducing not only the vegetable microbial counts but also common pathogens responsible for foodborne illness. Moreover, in previous studies using chitosan, the effect of the acid used as dissolvent is not often evaluated (as a control treatment) and in most of them only one acid is used so no comparisons of its antimicrobial activities can be made. Both acids used in the present study have significant antibacterial activity by themselves and when combined with chitosan (Conner and Kotrola 1995; Liu et al. 2006; No et al. 2002) and their effect should not be ignored.

Chitosan application on late development stages of lettuce (last 14 days before harvest) aims to the reduction of microbial population before the head is completely formed. In Butterhead lettuce, when the plant reach maturity, the head is comprised of a closely pack of leaves that encircle the inner leaves with the outer ones (Goñi et al. 2010). Any application of sanitizing solutions once the head is fully formed, will only reach the outer leaves, reducing its efficiency.

The antimicrobial activity of chitosan depended on several factors such as pH of the medium, temperature, and presence of several food components. 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 the microbial 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 (Kong et al. 2010).

In this sense, the effect of the different dissolvents on the antimicrobial activity of chitosan should be studied when applied in lettuces during their development. Therefore, the objective was to evaluate the effect of preharvest application of chitosan on native microflora of Butterhead lettuce at harvest and after 5 days of refrigerated storage (7–8 °C), using acetic and lactic acids as solvents.

Materials and methods

Chitosan solutions preparation

Chitosan powder (ACOFAR, Mar del Plata, Argentina; 98% deacetylation degree, 0.7% ash, 46 cP viscosity) was dissolved in acetic and lactic acids in the following concentrations: 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0% (w/v). Chitosan was reported to be soluble in dilute acidic solutions at pH below 6.0 (Toan et al. 2013). The pH was adjusted with a pH-meter (model 201, Hanna Instrumental, Portugal) to 5.6 with 0.1 M NaOH. Then, adjusted solutions were stirred for 2 h at room temperature at 100 rpm in an orbital shaker (TS-1000, Zhejiang, China).

Preharvest application of chitosan solutions

Butterhead lettuce heads (*Lactuca sativa* cv. Lores) were grown in Sierra de los Padres, Mar del Plata, Argentine. Lettuce heads were cultivated in greenhouses with mulch technology (a black plastic film separating each plant from the soil). The assays were performed in the fall—early winter.

Chitosan solutions were prepared as previously indicated, but only one concentration of chitosan was used: 10 g L^{-1}. It was dissolved in two different organic acids: 1% (w/v) acetic acid (AcH) and 0.8% (w/v) lactic acid (LacH). In the greenhouse, chitosan solutions were applied to each plant by spraying (7–10 mL/plant), taking care not to spread other plants. To avoid this dissemination, a plastic cone with an upper hole was place over each plant and the spray was applied inside the cone to avoid dispersion of the solution to nearby plants. Equal pressure was applied in each spray to ensure uniformity in the amount of solution applied to each plant. Three independent experimental runs were performed.

According to a previous applied protocol (Goñi et al. 2013b), chitosan solutions were applied in a SINGLE DOSE (14, 10, 7, 3 and 0 days previous to harvest) or in SUCCESSIVE DOSES (five applications at 14 + 10 + 7+3 + 0 days previous to harvest). Late development preharvest applications ensure that lettuce heads were still open, which allowed chitosan solutions to reach the inner leaves.

Lettuce heads were manually harvested, damaged external leaves were removed, then the cleaned heads were placed in polyethylene bags for transportation to the lab facilities (in less than 2 h). Once in the lab, lettuce heads were stored in a refrigerated chamber until processing (again no more than 2 h). To assess the effectiveness of the chitosan solutions on the postharvest microbial quality, a second batch of lettuce heads was stored at 7–8 °C and 98% relative humidity for 5 days.

Microbiological studies

For the analysis of microbial quality, 3 heads from each treatment were sampled at time zero and after 5 days of refrigerated storage (7–8 °C simulating commercial refrigeration). The enumeration of the microbial populations was made according to Ponce et al. (2008): mesophilic aerobic bacteria (MES) was performed in plate count agar (PCA) incubated at 30 °C for 48 h, psychrotrophic bacteria (PSC) in the same medium but incubated at 5–7 °C for 7 days, total coliforms (TC) in Mac Conkey agar incubated at 30–32 °C for 24 h, yeast and molds (YM) in yeast-glucose-chloranphenicol (YGC) medium incubated at 25 °C for 5 days. All culture medium were purchased from Britania, Buenos Aires, Argentina. Microbial counts were performed by duplicate in each of the 3 experimental runs (Ponce et al. 2008), and expressed as log CFU $g^{-1.}$

Statistical analysis

A completely randomized design was used and three independent runs were performed. Data obtained was analyzed with statistical software SAS 9.0 (SAS Inc., 2002). Results presented in the present work are lsmean values (means estimators by the method of least squares) and their standard deviations. Differences among treated samples were tested by variance analysis (ANOVA), PROC GLM (General Lineal Model Procedure) was used for the two-way ANOVA where factors employed were: application of the chitosan (TREATMENT) and the organic acid used to dissolve chitosan (ACID). For TREATMENT the levels were: 14, 10, 7, 3 or 0 days previous to harvest (dph) and a combination of all the latter (14, 10, 7, 3 and 0 dph, Successive). Control samples were comprised of Butterhead lettuce plants subjected to the same agricultural practices and the same environmental conditions, but without the application of chitosan solutions. One-way ANOVA, with only TREATMENT as factor, was applied to analyze the data obtained in the postharvest stage of the study. PROC UNIVARITE was used to validate the ANOVA assumptions in all stages and when significant differences were found, the Tukey–Kramer multiple comparison test was performed ($p < 0.05$).

Results and discussion

Preharvest application of chitosan dissolved in acetic and lactic acid: effect on Butterhead lettuce quality at harvest

In the present study, several acid concentrations were evaluated to assess stability of the chitosan solution (data not shown). Both, acetic and lactic acids were effective to solubilize chitosan at 5, 10 and 20 g L^{-1}, with concentrations higher than 0.7 and 0.8%, respectively. Therefore 1% (v/w) of acetic acid (as the traditional solvent of chitosan in previously reported studies) and 0.8% (v/w) of lactic acid (as the minimal concentration that was able to obtain a stable chitosan solution) were used for the in vivo application on the lettuce heads.

Preharvest application of chitosan represented a modified procedure that was required to be thoroughly studied before its final implementation. Moreover, extrinsic factors (weather conditions, agricultural practices or pests) and intrinsic interactions (food components, biofilm formation, and adherence of microflora to crevices in the surface of the leaf, among others) may be responsible for reducing antimicrobial activity in vivo as compared to in vitro. Table 1 shows the microbial counts (log CFU g^{-1}) of Butterhead lettuce at harvest, treated and untreated (control) with chitosan solutions when diluted in acetic acid 1% w/v (CHIT/acetic) or lactic acid 0.8% w/v (CHIT/lactic) at different development stages. No significant interactions TREATMENT*ACID were found for any of the microbial population studied, with p-values of 0.1965, 0.2077, 0.0998 and 0.3427 for MES, PSC, YM and TC, respectively. Results are presented for each single factor.

ACID was a significant factor for MES, YM and TC ($p < 0.05$). CHIT/acetic was more effective than CHIT/lactic in reducing YM ($p = 0.0461$) and TC ($p = 0.0004$) while CHIT/lactic was more effective in the reduction of MES ($p = 0.0049$). For PSC no significant effect was found when acetic acid was replaced with lactic acid ($p = 0.1773$). However, lettuce plants treated with CHIT/acetic solution presented symptoms of acid burn, even on application of single dose (Fig. 1). This was a clear example of the importance of in vivo validation of earlier in vitro results, especially when the test subject was a live organism like in this case with lettuce. Lettuce plants presented dried brown stains in their leaves which made them unacceptable from a sensorial point of view. These stains were not present in plants treated with CHIT/lactic, where no reduction of the sensorial quality was detected compared to untreated lettuces (data not shown).

On the other hand, TREATMENT was a highly significant factor for all microbial populations studied.

Table 1 Microbial counts (log CFU g^{-1}) of Butterhead lettuce at harvest, treated and untreated with chitosan solutions when diluted in acetic acid 1% w/v (CHIT/acetic) or lactic acid 0.7% w/v (CHIT/lactic)

MES		
	CHIT/acetic^{b}	CHIT/lactic^{a}
Control^{C}	6.65 ± 0.36	6.29 ± 0.55
Harvest^{AB}	5.16 ± 0.42	4.68 ± 0.62
3 dph^{C}	6.46 ± 0.44	5.86 ± 0.50
7 dph^{C}	6.42 ± 0.42	6.48 ± 0.49
10 dph^{C}	6.55 ± 0.51	6.02 ± 0.63
14 dph^{BC}	5.88 ± 0.36	5.83 ± 0.69
Successive^{A}	5.23 ± 0.44	3.66 ± 0.49
PSC		
	CHIT/acetic^{a}	CHIT/lactic^{a}
Control^{B}	5.77 ± 0.62	6.03 ± 0.65
Harvest^{A}	4.12 ± 0.49	5.07 ± 0.71
3 dph^{AB}	5.46 ± 0.55	5.88 ± 0.59
7 dph^{AB}	5.27 ± 0.48	5.94 ± 0.68
10 dph^{AB}	5.12 ± 0.61	5.48 ± 0.61
14 dph^{B}	5.63 ± 0.55	6.3 ± 0.75
Successive^{A}	5.15 ± 0.51	4.33 ± 0.79
YM		
	CHIT/acetic^{a}	CHIT/lactic^{b}
Control^{E}	6.68 ± 0.48	6.55 ± 0.51
Harvest^{AB}	4.42 ± 0.39	4.16 ± 0.49
3 dph^{CD}	5.32 ± 0.51	5.95 ± 0.60
7 dph^{CD}	5.08 ± 0.44	5.56 ± 0.55
10 dph^{BC}	4.64 ± 0.46	5.19 ± 0.48
14 dph^{DE}	5.33 ± 0.37	6.30 ± 0.42
Successive^{A}	4.02 ± 0.40	3.59 ± 0.45
TC		
	CHIT/acetic^{a}	CHIT/lactic^{b}
Control^{C}	5.16 ± 0.39	5.37 ± 0.52
Harvest^{AB}	3.09 ± 0.42	4.36 ± 0.58
3 dph^{AB}	3.76 ± 0.41	4.26 ± 0.62
7 dph^{C}	4.39 ± 0.38	5.44 ± 0.49
10 dph^{BC}	4.21 ± 0.33	4.96 ± 0.55
14 dph^{C}	4.81 ± 0.45	5.16 ± 0.61
Successive^{A}	3.21 ± 0.38	3.37 ± 0.52

No significant interactions were found, comparisons are made for single factors. Different letters in the same row (TREATMENT) or in the same column (ACID) indicate significant differences according to Tukey–Kramer multiple comparison test ($\alpha < 0.05$). Treatments: Control (plants without chitosan), Harvest (plants with chitosan solution applied right before harvest), Successive (plants with successive applications of chitosan during late stages of preharvest development), 3, 7, 10 and 14 dph (plants with chitosan solution applied 3, 7, 10 and 14 days before harvest, respectively)

MES mesophylic bacteria, *PSC* psicrophylic bacteria, *YM* yeasts and molds, *TC* total coliforms

Fig. 1 Butterhead lettuce plant at harvest, treated with chitosan solution in acetic acid 1% (w/v)

Single application and SUCCESSIVE applications were the most effective in reducing microbial counts at harvest when compared to control samples ($p < 0.005$). For HARVEST application, count reductions were 1.6, 1.3, 2.26 and 1.46 log, while for SUCCESSIVE applications, reductions were 2.0, 1.2, 2.8 and 1.9 log, for MES, PSC, YM and TC, respectively. As can be noted, YM were more susceptible to chitosan than bacteria, with higher reductions and almost every SINGLE DOSE application resulted in significant reductions compared to control (except for application 14 dph). As for SINGLE DOSE applied 3 dph, a significant reduction was found for YM and TC (1.1 and 1.2 log, respectively) while no significant effect was obtained for MES or PSC when compared to control.

In the first stage of the study, two conclusions were drawn: lactic acid did not significantly affect sensorial attributes of lettuce when applied in late stages of development maintaining its antimicrobial properties against PSC, increasing its activity against MES and only slightly reducing its antimicrobial activity against YM and TC. Despite the antimicrobial activity of chitosan dissolved in acetic acid, the noteworthy impact of the acetic acid solvent on the sensory quality of lettuce was sufficient reason for its removal in field trials. The second conclusion reached was that in order to reduce microbial counts at harvest, the most effective application moment of chitosan solutions was at HARVEST or in SUCCESSIVE applications.

More than 2 log reductions in MES and YM counts may be associated to an increase in shelf life, since they were commonly referred to as the main cause of spoilage of fresh-cut lettuce during postharvest storage. YM are source of deleterious effects in lettuce leaves due to the apparition of rotting. In addition, a 99% reduction was obtained for TC (when CHIT/lactic was applied in successive doses). This by itself represents an improvement in the vegetable safety, improving the microbiological quality of the lettuce plants at harvest.

Chitosan is known for its ability to form a barrier that control gas exchange and reduces water loss, which contributes to maintaining tissue integrity and reduces microbial decay (Devlieghere et al. 2004; Dong et al. 2004). Applying CHIT/lactic to lettuce heads could result in a thin film that may reduce the ability of the microorganisms to successfully attach to the plant.

Concluding, CHIT/lactic should be applied 3 dph, at HARVEST or in SUCCESSIVE applications (14, 10, 7, 3 and 0 dph) to achieve maximum reduction of native microflora at harvest, especially in YM and TC. Since no significant effect on native microflora was detected with the remaining single doses (14, 10 or 7 dph) those treatments were excluded from the second part of this study (postharvest evaluation) which was carried out only for 3 dph, HARVEST and SUCCESSIVE applications of CHIT/lactic solutions.

Replacement of acetic by lactic acid in the preharvest application of chitosan: effect on Butterhead lettuce quality during postharvest storage

In order to establish if chitosan antimicrobial effect at harvest remained during storage, lettuce heads treated with CHIT/lactic were stored at 7–8 °C, a non-restrictive temperature for microbial growth frequently used in supermarkets and domestic fridges. The microbial counts of Butterhead lettuce, treated with a CHIT/lactic at late stages of development (preharvest), after 5 days of refrigerated storage are shown on Fig. 2.

Only those lettuce heads treated with SUCCESSIVE applications of CHIT/lactic presented significant reductions in all the microbial populations evaluated. Preharvest application of CHIT/lactic was able to reduce YM and TC counts, with 2.0 and 1.8 log reductions when compared to control, respectively. TC are not necessarily harmful themselves; still, they are considered as an indicator of product safety. YM are frequently related to quality loss of fresh vegetables during postharvest storage due to rotting and the development of undesirable color stains and off-odors that cause economic loses.

Lettuce plants treated with successive applications showed significant reductions in MES and PSC (Fig. 2) with 1.1 log and 1.4 log reductions, respectively. After 5 days of refrigerated storage the preharvest application of CHIT/lactic on single dose was unable to maintain the effect observed at harvest on any of the microbial populations studied.

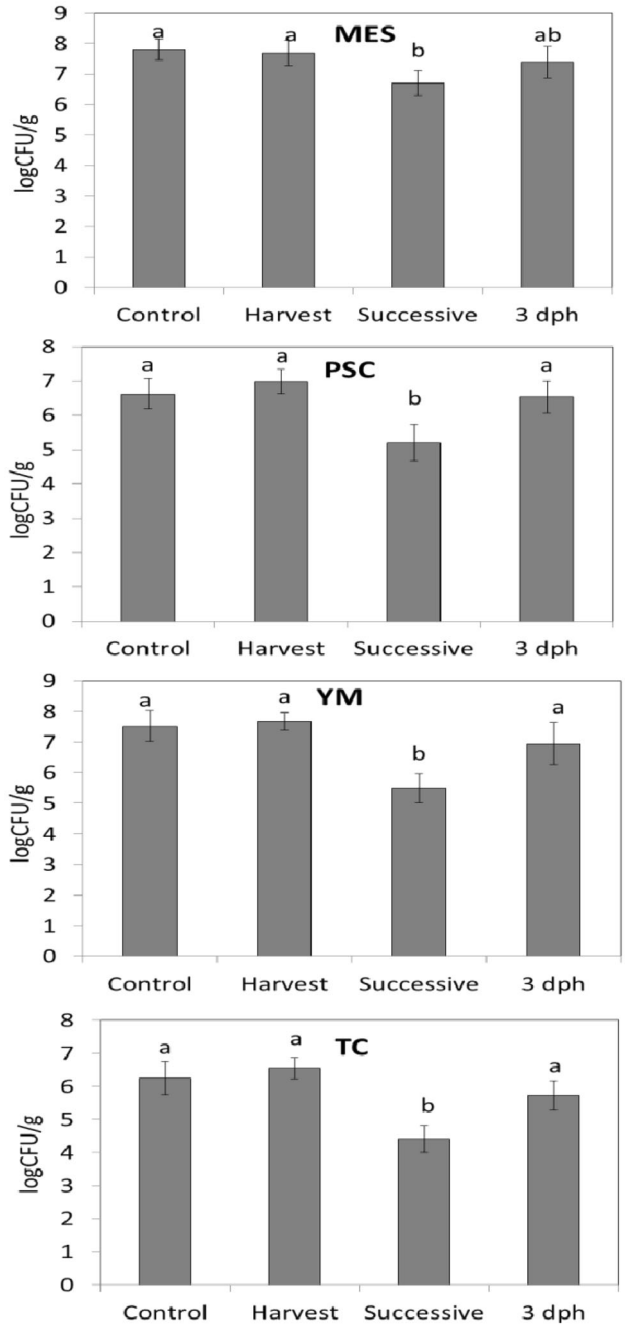

Fig. 2 Microbial counts (log CFU/g) of Butterhead lettuce treated during preharvest with chitosan diluted in lactic acid 0.8% v/v, after 5 days of refrigerated storage (7–10 °C). References: *MES* mesophylic bacteria, *PSC* psicrophylic bacteria, *YM* yeasts and molds, *TC* total coliforms. Treatments: Control (plants without chitosan), Harvest (plants with chitosan solution applied right before harvest), Successive (plants with successive applications of chitosan during late stages of preharvest development), 3 dph (plants with chitosan solution applied 3 days before harvest)

The results exposed here present enough evidence indicating that after successive chitosan applications on late stages of lettuce development, the plants improved their microbiological quality at harvest and also acquire enhanced resistance to postharvest microorganism growth during refrigerated storage. This may indicate that the use of natural elicitors such as chitosan might assist in the goal of improving the microbiological quality of fresh-cut leafy vegetables.

Conclusions

Replacement of acetic acid for lactic acid did not affect the antimicrobial activity of chitosan against native microflora of Butterhead lettuce when applied in late stages of development. Moreover, lactic acid did not produced brown stains in the leaves like acetic acid did, reducing sensorial quality to unacceptable values. A successive preharvest application of CHIT/lactic (10 g L^{-1} in a 0.8% lactic acid solution) on late stages of development (14, 10, 7, 3 and 0 days before harvest) reduced in almost 99% YM and TC population, not only in freshly cut lettuce but also during postharvest refrigerated storage.

Safe fresh produce begins in the farm and Good Agricultural Practices (GAP) coupled with Good Manufacture Practices (GMP) during the commercialization chain should be applied to reduce economical loses and lower the risk of illnesses associated to food borne pathogens. Produce that is grown and sold with little biological contamination is less likely to result in health hazards caused by poor handling during later processing stages. Preharvest application of CHIT/lactic could easily be introduced in a GAP routine, without consequences on the environment since chitosan and lactic acid are both Generally Recognize as Safe (GRAS) substances.

Acknowledgements This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANCyT) and Universidad Nacional de Mar del Plata (UNMDP).

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