Effect of Lactoperoxidase System on Microbiological and Quality Markers of Minimally Processed Lettuce

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Abstract

Minimally processed lettuce is sensitive to microbial attack caused by the loss of natural resistance and their high water content and nutrients. The use of natural anti-microbial agents such as the lactoperoxidase system (LPS) represents an interesting alternative to the use of chemical treatments. This study focused on the importance of LPS and its efficiency as a bioprotective agent. The effect of washing with LPS, at two different concentrations (4 and 5 IU) and four different treatment times (1, 2, 3 and 4 h) on microbial growth in fresh-cut lettuce, was determined at 30˚C and compared with a 80 ppm chlorine at 4˚C for 20 min. The results deduced that both doses of LPS showed significant reductions in microbial growth when applied during 4 h, but the highest dose proved to be more effective. No significant differences were found between the LPS treatment and chlorine considering native microflora reduction and quality markers during storage.

Keywords

Antimicrobial Effect, Lactoperoxidase System, Minimally Processed Lettuce, Quality Markers

1. Introduction

Minimally processed lettuce form an important component of a healthy diet and is a convenient way to increase the consumption of this fresh vegetable [1]. This fresh product, however, is sensitive to microbial attack after harvest caused by the loss of natural resistance and their high water content and nutrients, a problem that can be worsened by minimal processing. To maintain the microbiological quality and safety of minimally processed fresh vegetables, these products

are normally packaged in modified atmospheres and effective refrigerated temperature control during manufacture, distribution and retailing are required. Unfortunately, these steps do not remove either or retard microbial spoilage of these products entirely [1] [2]. The dominant bacterial population of lettuce at low temperatures during storage consists mainly of species.

Disinfection using hypochlorite is currently used by a majority (76%) of fresh produce manufacturers to enhance safety and shelf-life profiles. Water containing 50 - 200 ppm of chlorine is used extensively in food processing plants to disinfect whole fruits and vegetables as well as fresh-cut produce. However, chlorine does not ensure elimination or even an efficient reduction in the number of bacteria [3]. In addition to such limited efficacy, chlorinated organic compounds, such as trihalomethanes, can be produced when the chlorine contacts the organic matter. Due to these problems, new awareness to reduce the usage of the chemical treatments by developing alternative strategies or technologies in order to improve fresh cut vegetables disease resistance and control of pathogens are being promoted. Biological control well suited for this new trend and Lactoperoxidase system (LPS) has been identified as bioprotective agents [4].

LPS name points out the dairy origin [5]. This system consists of the lactoperoxidase enzyme and two substrates: the hypothiocyanate ion ($OSCN^-$) and hydrogen peroxide (H_2O_2). The antimicrobial element of LPS, namely the hypothiocyanate ion ($OSCN^-$), is the reaction between thiocyanate and hydrogen peroxide, catalysed by lactoperoxidase [6]. Antimicrobial activity of this system has been shown for a number of bacteria, fungi and viruses [5] [7].

The main objective of this work was to optimize the application of lactoperoxidase system (LPS) for minimally processed lettuce decontamination. Moreover, the effect of the LPS system on the control of spoilage microflora and improving shelf-life characteristics during lettuce storage was determined.

2. Materials and Methods

2.1. Lactoperoxidase System

Lactoperoxidase enzyme (200 IU/mg) was donated by Sigma-Aldrich (France), glucose oxidase (Sigma-Aldrich, France) and glucose were used as a peroxide generator system and sodium thiocyanate (NaSCN) were purchased from Sigma-Aldrich (France).

2.2. Preparation of Vegetable Model Lettuce

Biological iceberg lettuce were harvested at optimal maturity. Samples were then transferred to the laboratory within 1 h under refrigeration conditions and stored up to 24 h at 4°C before treatment. Three external leaves were removed by hand (using sterile gloves) and the other parts of the lettuce were shredded in pieces of approximately 2 cm^2, using a sterile blade. Three separate treatment solutions were prepared using distilled water. The concentrations were 4 and 5 IU for LPS, 80 ppm for chlorine and temperatures were 30 and 4°C respectively

[8]. Prepared lettuce was placed in the appropriate treatment solution with gentle agitation during 20 min for chlorine and 1, 2, 3 and 4 h for LPS, prior to rinsing in distilled water for 1 min. The ratio of samples to treatment solution was 1:10 w/v. Treated lettuces were rinsed with distilled water for 1 min [8]. They were then spin-dried, divided into 10 g of portions and placed into plastic bags. All bags were treated as individual samples and stored at 4˚C. Unwashed samples and samples treated with chlorine water were used as controls for Shredded lettuce washed with LPS.

2.3. Microbiological Analysis

Microbiological analysis was performed on hour, 1, 2, 3 and 4 to determine the effect of LPS concentration and contact time applied.

The enumeration of the microbial population was made according to Guireaud *et al.* (1998) [9]. Each 10 g of lettuce was washed in a bag containing 90 mL of sterile peptone water with shaking. The homogenate was diluted decimally in peptone water. 1 mL of the diluted samples were transferred on plate count agar (PCA) (Merck, Darmstadt, Germany) to enumerate mesophilic aerobic bacteria (MES) and incubated at 30˚C for 48 h. Psychrotrophic bacteria (PSC) was performed in the same medium but incubated at 6˚C for 5 - 7 days. Total coliforms (TC) in Desoxycholate 1‰ agar (Merck, Darmstadt, Germany) incubated at 37˚C for 24 h. Lactic acid bacteria (LAB) in Man, Rogosa and Sharpe agar (MRS) (Merck) incubated at 37˚C for 48 h. Molds and yeast (MY) in Sabouraud Chloramphenicol agar (Merck, Darmstadt, Germany) incubated at 25˚C for 5 days. Each analysis was carried out in triplicate. Results were expressed as $\log_{10}$ CFU g^{-1}.

To investigate the LPS effect on the preservation of fresh lettuce, samples were stored in refrigerator (4˚C) 7 days and microbiological analyses were conducted on treatment day and on 1st, 2nd, 3rd, 5th and 7th days of the refrigerated storage.

2.4. Shelf-Life Analysis

The objective was to study the effect of LPS on the quality of minimally processed lettuce. Weight loss, colour and pH were evaluated as indicators of shelf-life. Shelf-life is defined as the length of time at which the vegetable can maintain the appearance and safety [10], therefore markers were measured from samples treated with LPS and compared to those obtained using chlorine on days 0, 1, 2, 3, 5 and 7.

2.4.1. Weight Loss

Samples were weighed immediately after treatment (*IW*, initial weight) and then, after removal from refrigerated storage (*SW*, storage weight). Weight loss of each individual sample was calculated as:

$$WL(\%) = (1 - SW/IW) \times 100$$

WL was expressed as percentage of weight loss with respect to initial mass.

2.4.2. Colour Measurement

Color measurements of lettuce were performed using a colorimeter (Minolta CR-300, Japan) to measure the CIE color space co-ordinates, L*, a* and b* (lightness, red value and yellow value, respectively, on the Hunter scale). The colorimeter was calibrated with a white tile standard.

2.4.3. pH

Ten grams of lettuce was blended for 2 min in 20 mL of deionised water. The pH of the slurry was determined at room temperature using a pH-meter (Hach Company Electrochemical Meters).

2.5. Statistical Analysis

The mean values obtained from the microbiological evaluation of lettuce were analyzed by one one-way ANOVA procedure of SPSS® 17.0. Duncan's multiple range tests were used to determine any significant difference between mean values and evaluations as based on a significance level of $p < 0.05$.

3. Results and Discussion

3.1. Effect of LPS Concentration and Time Contact on the Natural Microflora of Lettuce

Table 1 shows microbial counts in shredded lettuce after treatment with LPS and Chlorine. The effect of LPS after 1 h of treatment, on MES, PSC, TC, and YM was significantly different ($p < 0.05$) to unwashed samples. For LAB, significant reductions were obtained with dose application of 5 IU after two hours. The effect of the LPS can be bactericidal against the Gram negative bacteria or bacteriostatic against Gram-positive bacteria [7] (Marshall and Reiter, 1980). In

Table 1. Survival of MES, PSC, TC, LAB and YM on minimally processed lettuce treated with LPS (doses 4 and 5 IU) and chlorine.

Treatment	MES	PSC	TC	LAB	YM
NTC	6.5 ± 6.2^A	4.4 ± 4.2^A	4.9 ± 3.9^A	5.3 ± 4.7^A	5.9 ± 4.2^A
1 LPS 1	6.0 ± 4.8^B	4.3 ± 4.2^B	4.7 ± 4.1^B	5.3 ± 4.7^A	5.6 ± 4.2^B
1 LPS 2	6.0 ± 4.8^{BC}	4.0 ± 1.8^B	4.0 ± 3.0^C	5.2 ± 4.8^A	5.1 ± 1.8^C
2 LPS 1	5.9 ± 4.6^{CD}	4.1 ± 3.8^C	4.4 ± 4.2^D	5.2 ± 4.6^A	4.9 ± 3.8^{CD}
2 LPS 2	5.7 ± 4.8^E	3.1 ± 2.2^D	3.9 ± 3.2^E	4.2 ± 3.7^{BC}	4.2 ± 2.2^D
3 LPS 1	5.9 ± 4.6^D	4.0 ± 3.4^C	3.7 ± 3.0^F	4.9 ± 4.2^D	4.0 ± 3.4^D
3 LPS 2	4.1 ± 3.3^F	2.9 ± 2.0^{EF}	2.9 ± 2.0^G	4.0 ± 2.8^{BC}	3.0 ± 2.0^E
4 LPS 1	5.6 ± 5.4^E	3.0 ± 1.8^{DE}	2.3 ± 2.0^H	4.4 ± 4.2^B	3.7 ± 1.8^F
4 LPS 2	3.6 ± 2.9^G	ND^G	ND^I	3.8 ± 3.0^C	2.2 ± 0.0^E
Chlorine	3.9 ± 3.1^H	2.8 ± 2.0^F	ND^I	2.4 ± 1.9^C	1.8 ± 2.0^E

MES: total mesophyllic bacteria count, PSC: total psychrophilic bacteria count, TC: total coliforms, LAB: lactic acid bacteria and YM: total yeast and molds count. Counts are expressed in Log_{10} CFU g^{-1} (±standard deviation). Different letters in the same column indicate significant differences ($p < 0.05$). The concentrations used for each treatment were the following: LPS 1 (4 IU), LPS 2 (5 IU) and chlorine (80 ppm). The number used before LPS is the number of hours of treatment. NTC: non-treated control.

fact, this difference in sensitivity can probably explained by the differences in cell wall structure and their different barrier propreties [11]. The inner membrane of Gram positive bacteria appears to be less extensively damaged by LPS treatment that than of Gram negative species [7].

Changes in the mean values of CFU g^{-1} in Log_{10}, the main groups of lettuce contaminant bacteria, measured at 4 h post activation of LPS, indicate the best decrease of the total amount, independently of the applied dose. For example, reduction of YM with 0.3, 1, 1.9 and 2.2 log units were obtained after 1, 2, 3 and 4 h of treatment with the lowest dose of LPS (4 IU), respectively. These results confirm a previous report in which LPS was effective on reducing microorganisms growth in vegetable juices, the reduction becomes important after 4 h of contact [12].

The use of two doses of LPS has been determinant for the effective selection of appropriate concentration, since for all microbial group differed between 4 and 5 IU of lactoperoxidase used for 4 h. Indeed, the best reductions were observed for the higher dose (5 IU).

When LPS was used for 4 h, the effect on TC, LAB and YM was the same as that observed with the Chlorine ($p > 0.05$). For MES and PSC, a significant inhibitory effect was found with LPS in comparison to the chlorinated water. Thus, from a microbiological point of view, LPS is a viable alternative to chlorine as decontamination treatments.

Sagoua *et al.* (2011) [13] showed that the LPS had a significant inhibitory effect on the microbial growth. Nguyen *et al.* (2005) [14] also showed the effect of LPS on the growth of two mango pathogens, *Colletotrichum gloeosporioides* and *Botryodiplodia theobromae*. They demonstrated that the inhibitory effect was due to $OSCN^-$ ions produced by the lactoperoxidase system and not to the hydrogen peroxide present in the system. In these experiments, the source of hydrogen peroxide was glucose oxidase in salt solutions.

Finally, we selected 5 IU for lactoperoxidase dose and 4 h of contact for the rest of our study.

3.2. Effect of LPS and Chlorine on the Natural Microflora of Lettuce during Storage

Reduction of the indigenous flora of lettuce because of decontamination with watery suspensions of LPS and chlorine are shown in Table 2. Washing of two lettuce samples in the different water (LPS and chlorine) resulted in similar reduction of indigenous flora in both samples.

If decontamination of lettuce sample was performed with LPS solution a reductions of 3.3, 4.9 4.6, 3.1 and 3.6 Log_{10} CFU g^{-1}, respectively, were observed for MES, PSC, TC, LAB and YM. These reductions were important throughout the cold storage. The sensibility of MES and PSC to LPS has been previously cited for Björck (1978) [15] who showed that the numbers of these bacteria decreased markedly in the raw milk, inter alia, growth of this microbial population was strongly inhibited by the presence of LPS. Similarly, the publications of the

Table 2. Survival of MES, PSC, TC, LAB and YM on minimally processed lettuce treated with LPS (5 IU) and chlorine during storage (0 - 7 days at 4˚C).

Time (days)	Bacterial population	Treatment		
		LPS	*Chlorine*	*NTC*
0	MES	3.5 ± 3.5^A	3.8 ± 3.8^A	6.8 ± 6.0^B
	PSC	ND^A	1.1 ± 0.8^A	4.9 ± 4.2^B
	TC	ND^A	ND^A	4.6 ± 4.2^B
	LAB	1.6 ± 1.2^A	1.5 ± 1.0^A	4.7 ± 3.8^B
	YM	2.1 ± 1.8^A	1.1 ± 0.8^A	5.7 ± 5.7^B
1	MES	3.5 ± 3.5^A	3.8 ± 3.8^A	8.6 ± 8.6^B
	PSC	1.4 ± 0.8^A	1.5 ± 1.0^A	4.8 ± 4.5^B
	TC	ND^A	ND^A	4.6 ± 4.1^B
	LAB	1.7 ± 1.3^A	1.7 ± 1.5^A	5.7 ± 5.5^B
	YM	2.2 ± 2.1^A	1.7 ± 1.4^A	5.8 ± 5.7^B
2	MES	3.8 ± 3.6^A	4.1 ± 3.9^A	8.7 ± 8.6^B
	PSC	2.5 ± 2.2^A	2.0 ± 1.0^A	4.5 ± 4.0^B
	TC	ND^A	ND^A	4.7 ± 4.1^B
	LAB	2.9 ± 2.3^A	3.6 ± 3.5^A	6.8 ± 6.4^B
	YM	2.5 ± 1.8^A	1.7 ± 1.4^A	6.6 ± 6.7^B
3	MES	4.2 ± 3.9^A	4.2 ± 4.1^A	10.0 ± 9.5^B
	PSC	3.7 ± 3.3^A	3.1 ± 2.2^A	6.4 ± 5.8^B
	TC	ND^A	ND^A	5.0 ± 4.0^B
	LAB	2.8 ± 2.4^A	2.9 ± 2.5^A	7.7 ± 7.6^B
	YM	2.8 ± 2.4^A	3.7 ± 3.6^A	6.4 ± 6.4^B
5	MES	5.5 ± 5.6^A	5.6 ± 5.6^A	10.8 ± 10.9^B
	PSC	5.8 ± 5.2^A	5.4 ± 5.2^A	7.7 ± 7.3^B
	TC	1.0 ± 1.0^A	1.0 ± 1.0^A	5.9 ± 5.4^B
	LAB	3.9 ± 3.4^A	3.9 ± 3.4^A	8.6 ± 7.8^B
	YM	4.9 ± 3.8^A	4.6 ± 4.2^A	8.5 ± 8.6^B
7	MES	7.8 ± 7.3^A	7.6 ± 7.4^A	12.2 ± 11.6^B
	PSC	6.6 ± 6.6^A	6.6 ± 6.5^A	8.7 ± 8.2^B
	TC	2.3 ± 2.3^A	2.5 ± 2.3^A	7.7 ± 7.5^B
	LAB	4.9 ± 4.0^A	4.8 ± 4.4^A	10.8 ± 10.1^B
	YM	5.8 ± 5.5^A	5.6 ± 5.3^A	8.8 ± 8.3^B

MES: total mesophyllic bacteria count, PSC: total psychrophilic bacteria count, TC: total coliforms, LAB: lactic acid bacteria and YM: total yeast and molds count; *LPS*, *Chlorine*: lettuce samples treated by the LPS and chlorine respectively; NTC: non-treated control; ND: No detected; Values are means of three replicates and counts are expressed in Log_{10} CFU g^{-1} (±standard deviation); Means followed by different letters are significantly different ($p < 0.05$) for each bacterial population.

French Food Safety Agency [8] described the opinion concerning the authorization of using a lactoperoxidase system as a processing aid for the treatment of fresh-cut, ready-to-eat salads. This process actually seems to have the same effect as chlorine as a processing aid, in fresh-cut, ready-to-eat production conditions, and this for the microbiological indicators used in this sector (mesophilic aerobic flora), providing the operating conditions are the same as in the submitted file [16]. Our results also reported that MES were affected by chlorine as there were affected by the LPS.

The mean aerobic mesophilic counts were 5.5 and 5.6 Log_{10} CFU g^{-1} for LPS and chlorines samples respectively after 5 days of storage, indicating that all

treated samples were acceptable for consumption because the aerobic bacterial count for lettuce fresh is less than 7.7 Log_{10} CFU g^{-1} [17].

3.3. Shelflife Analysis

3.3.1. Weight Loss

Fresh-cut lettuce is very susceptible to weight loss which is due to moisture loss. Yet, the relative humidity is generally very high inside the bags, so dehydration is not a common problem when lettuce are packaged [18]. As the weight loss is an indicator of quality retention, this was monitored during storage for this fresh-cut vegetable. No significant ($p > 0.05$) decrease in weight loss was observed during the first two days in both treated samples, with significant differences between treatments and control (Table 3). Samples treated with the chlorine showed higher weight loss than samples treated with LPS from day 3 to 7. This weight loss corresponds to the natural catabolism process in vegetable commodities, catalysed by enzymes and accelerated by the minimal processing [19].

3.3.2. Colour

Colour was monitored in all the samples during the entire storage (Table 3). Luminosity values (L*) decreased during storage, and this can be explained by the appearance of browning. Compared with a control treatment (chlorine), LPS samples showed similar values in luminosity ($p > 0.05$) during the 5 first days of storage.

Another colour parameter related to browning [20] (Castaner *et al*. 1999) and to the degradation of chlorophyll [21] is a* value. This Redness/greenness parameter (a*) significantly ($p < 0.05$) increased during the storage. These decreases in green colour were coupled with an increase in red colour (b*) responding to the browning appearance.

3.3.3. pH

The initial pH of the lettuce is 6.52 (Table 3), but as reported by Seifu *et al*. (2005) [22] the optimum pH for LPS activity is comprised inter 5 and 6. The enhanced bactericidal effect of this system at low pH may be due mainly to protonation of the oxidation products. At acidic pH, the major oxidation product is known to be hypothiocyanous acid (HOSCN, pKa 5.3). HOSCN, being uncharged, could more readily diffuse through the hydrophobic bacterial membrane than the hypothiocyanate ion to oxidize intracellular components [23]. Nonetheless, after 4 h exposure to the LPS, the enhanced bactericidal effect of the system can be improved because of the accumulation of the oxidation products [12].

A general decrease of pH was observed over storage, which could be due to an increase in the bacterial growth [24] [25]. Samples treated with LPS showed highest pH values from day 1 to 7 than samples treated with the chlorine.

4. Conclusions

The results showed that the variable time of exposure to different LPS doses

Table 3. Effect of storage and treatment (LPS and chlorine) on pH, weight loss (%) and colour in fresh-cut lettuce stored at 4°C for 7 days.

Time (days)	Parameter	Treatment		
		LPS	*Chlorine*	*NTC*
0	pH	6.38 ± 0.01[A]	6.25 ± 0.09[B]	6.52 ± 0.09[C]
	WL	0.00 ± 0.00[A]	0.00 ± 0.00[A]	0.00 ± 0.00[A]
	L	61.71 ± 1.15[A]	64.59 ± 2.17[A]	64.43 ± 1.11[A]
	a	−16.39 ± 0.47[A]	−15.54 ± 0.03[A]	−16.19 ± 1.86[A]
	b	30.60 ± 0.01[A]	28.91 ± 0.30[B]	30.44 ± 0.28[A]
1	pH	6.36 ± 0.17[A]	6.21 ± 0.06[B]	6.35 ± 0.76[A]
	WL	0.66 ± 0.01[A]	0.82 ± 0.08[A]	5.51 ± 0.25[B]
	L	61.89 ± 0.10[A]	63.67 ± 0.10[A]	59.79 ± 0.10[B]
	a	−16.28 ± 0.27[A]	−14.07 ± 0.55[B]	−15.03 ± 1.55[AB]
	b	30.05 ± 0.54[A]	28.30 ± 0.91[B]	30.12 ± 0.44[A]
2	pH	6.33 ± 0.10[A]	6.13 ± 0.30[B]	6.08 ± 0.44[B]
	WL	2.72 ± 0.01[A]	3.41 ± 0.36[A]	11.45 ± 0.78[B]
	L	60.05 ± 0.10[A]	60.05 ± 0.10[A]	54.61 ± 0.10[B]
	a	−16.20 ± 0.13[A]	−12.97 ± 0.70[B]	−14.69 ± 0.38[C]
	b	29.63 ± 0.33[A]	27.49 ± 0.50[B]	28.93 ± 0.13[A]
3	pH	6.32 ± 0.08[A]	6.10 ± 0.09[B]	5.84 ± 0.09[C]
	WL	4.47 ± 0.12[A]	5.84 ± 0.56[B]	15.07 ± 0.57[C]
	L	59.72 ± 0.41[A]	57.90 ± 1.48[A]	49.89 ± 1.02[B]
	a	−13.15 ± 1.01[A]	−12.55 ± 1.19[A]	−13.43 ± 1.05[A]
	b	28.82 ± 0.42[A]	25.43 ± 0.56[B]	27.26 ± 0.69[C]
5	pH	6.24 ± 0.08[A]	6.02 ± 0.08[B]	5.30 ± 0.08[C]
	WL	8.82 ± 0.18[A]	11.88 ± 0.11[B]	16.87 ± 1.70[C]
	L	56.38 ± 1.80[A]	55.86 ± 1.46[A]	44.59 ± 0.06[B]
	a	−11.49 ± 1.41[A]	−10.52 ± 0.77[A]	−11.83 ± 0.62[A]
	b	26.50 ± 0.40[A]	24.20 ± 0.26[B]	25.21 ± 0.25[C]
7	pH	6.20 ± 0.08[A]	6.00 ± 0.08[B]	5.05 ± 0.08[C]
	WL	10.43 ± 0.67[A]	12.64 ± 0.51[B]	16.30 ± 1.08[C]
	L	51.15 ± 0.17[A]	53.39 ± 1.45[B]	38.96 ± 1.07[C]
	a	−10.16 ± 0.89[A]	−10.10 ± 0.27[A]	−11.79 ± 0.83[B]
	b	25.27 ± 0.25[A]	22.50 ± 0.52[B]	23.72 ± 0.62[C]

pH, WL, L, a, b: pH, weight loss, colour parameter; *LPS*, *Chlorine*: lettuce samples treated by the LPS and chlorine respectively; NTC: non-treated control; Values are means of three replicates and counts are expressed in Log_{10} CFU g^{-1} (±standard deviation); Means followed by different letters are significantly different (p < 0.05) for each bacterial population.

affected most of the microbial quality. Based on this quality tested 4 h and 5 IU can be considered the best time and the best dose for the application for keeping fresh-cut lettuce microbial quality. The effectiveness of LPS as a decontamination treatment was comparable with that of chlorine. Application of LPS according to the proposed protocol were effective in inhibiting the growth and survival of spoilage microorganisms associated to minimally processed vegetables, without affecting its Quality markers.

However, in order to increase and improve the efficiency of LPS, coupling with other substances with antimicrobial potential can be considered.

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