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Original Article

Inactivation of *Salmonella Enteritidis* on lettuces used by minimally processed vegetable industries

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Abstract
Introduction: Washing and disinfection methods used by minimally processed vegetable industries of Southern Brazil were reproduced in laboratory in order to verify their effectiveness to reduce *Salmonella* Enteritidis SE86 (SE86) on lettuce.
Methodology: Among the five industries investigated, four carried out washing with potable water followed by disinfection with 200 ppm sodium hypochlorite during different immersion times.
Results: The washing procedure alone decreased approximately 1 log CFU/g of SE86 population and immersion times of 1, 2, 5, and 15 minutes in disinfectant solution demonstrated reduction rates ranging from 2.06±0.10 log CFU/g to 3.01±0.21 log CFU/g. Rinsing alone was able to reduce counts from 0.12±0.63 log CFU/g to 1.90±1.07 log CFU/g. The most effective method was washing followed by disinfection with 200 ppm sodium hypochlorite for 15 minutes and final rinse with potable water, reaching 5.83 log CFU/g of reduction. However, no statistical differences were observed on the reduction rates after different immersion times.
Conclusion: A time interval of 1 to 2 minutes may be an advantage to the minimally vegetable processed industries in order to optimize the process without putting at risk food safety.

Key words: Minimally processed vegetable industries; washing and disinfection; sodium hypochlorite; *Salmonella Enteritidis* SE86.

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Introduction

Vegetable and fruits are recommended as an important dietary source of micronutrients, vitamins, and fibers for humans and are vital for health and well-being [1]. Their consumption has dramatically increased in the past years worldwide, at the same time that increased the number of foodborne diseases associated to these types of foods [2]. Among the most important pathogens associated with fresh products are *Salmonella* spp. strains, which have been responsible for several foodborne outbreaks [3-6].

Each year, food contaminated with *Salmonella* causes an estimated 1.3 million illness cases and approximately 500 deaths. Preventing *Salmonella* infections depends on actions taken to reduce food contamination by regulatory agencies, food industries, and consumers, as well as actions taken for detecting and responding to the outbreaks when they occur [7]. In Brazil, between 2000 and 2014, 38.2% of reported foodborne outbreaks were caused by *Salmonella* spp. [8]. And a specific strain of *Salmonella* Enteritidis (SE86) have been identified in several Salmonellosis outbreaks since 1999 to 2013, in the State of Rio Grande do Sul (RS), Southern Brazil [9,10]. This has been one of the most studied foodborne pathogens in Southern Brazil during the last 15 years [10].

During the last decade, the number of minimally processed vegetable industries has considerably increased in Brazil. In these types of industry, microbial contamination present on vegetables is typically reduced through washing and sanitization procedures [11], which correspond to the main critical points of processing [12]. According to the Codex Alimentarius, minimally processed vegetable industries can use different methods and products for disinfection [13], and chlorine compounds seems to be the preferred option because they are cheap and present broad-spectrum microbial inactivation [14]. Currently, in Brazil, there is no official regulation recommending sanitizers or how to wash and disinfect vegetables in minimally processed vegetable industries, and several methods have been used without a comparison regarding its effectiveness against important food pathogens.

The objective of this study was to evaluate the inactivation of *Salmonella* Enteritidis SE86 on lettuces processed to different methods of washing and disinfection used by industries of minimally processed vegetables of Southern Brazil.

Methodology

Investigation of processing characteristics

The processing characteristics of five companies of minimally processing fruits and vegetables located in the State do Rio Grande do Sul, Southern Brazil, were followed. As a pre-requisite to participate of this study, all the included food businesses signed an agreement of cooperation with the Division of Sanitary Surveillance of the State of Rio Grande do Sul. Each industrial plant was visited two times, between October 2013 and January 2014 by an Inspector of the Division of Sanitary Surveillance of the State of Rio Grande do Sul who collected information about processing. Washing and disinfection steps of each company were carefully accompanied and reproduced in the Laboratory of Food Control and Food Microbiology – ICTA/UFRGS in order to verify the effectiveness on the inactivation of SE86 on lettuces. The characteristics of each process are described in Table 1.

The washing and rinsing steps were reproduced at laboratory using 250 mL of potable water. A commercial brand of sanitizer (Água Sanitária Qboa, Porto Alegre, Brazil), usually found in industries, was chosen to perform the tests, being the immersion conducted in 500 mL of 200 ppm of this sanitizer.

Experimental design

Salmonella Enteritidis SE86 was used as inoculum. The strain was originally isolated from a cabbage responsible for a foodborne outbreak occurred in 1999 in Southern Brazil and was responsible for more than 90% of Salmonellosis identified in the State of RS during the last years [9,10].

Before the artificial contamination of the lettuce leaves, SE86 strain was kept at -20°C in 30% glycerol. In the day before inoculation, SE86 was subcultivated in Brain Heart Infusion (BHI) (HiMidea, Mumbai, India), for 24 hours at 37°C. Decimal dilutions were done using 0.1% peptone water (HiMidea, Mumbai, India) in order to reach a final concentration of 10^6 CFU/g.

Curly lettuces were purchased from local markets and transported to the laboratory. Before the analysis, outer leaves and core were removed from the lettuces heads and the absence of *Salmonella spp.* was confirmed by the ISO 6579:2002 [15]. Lettuce leaves were not washed or disinfected before inoculation in order to simulate real conditions. Samples of internal leaves weighting 10 g were added to sterile plastic bags and inoculated with 100 µL SE86 suspension, reaching a final concentration of 10^5 – 10^6 CFU/g. Inoculated lettuces were let for 30 minutes at room temperature and processed according procedures described in Table 1. Each procedure was repeated two times with three replicates per trial.

After each step of each procedure, lettuce samples were added to a new sterile plastic bag containing 90 ml of 0.1% peptone water added with 0.9% NaCl. Each sample was homogenized using a stomacher (Seward, London, UK) for 30 seconds and was serially diluted in

Table 1. Washing and disinfection processes used at different minimally processing vegetable industries of Southern Brazil which were reproduced in the laboratory in order to inactive *Salmonella* Enteritidis SE86 on lettuce.

	Identification	**Procedure**
	Washing[a]	a
Industry 1	Washing followed by immersion[b] in 200 ppm of sodium hypochlorite for 2 minutes	b
	Washing followed by immersion[b] in 200 ppm of sodium hypochlorite for 2 minutes and rinsing[c]	c
Industry 2	Immersion[b] in 200 ppm of sodium hypochlorite for 15 minutes	d
	Immersion[b] in 200 ppm of sodium hypochlorite for 15 minutes followed by immersion[b] in coconut soap for 1 minute	e
	Immersion[b] in 200 ppm of sodium hypochlorite for 15 minutes followed by immersion[b] in 10% coconut soap for 1 minute and immersion[b] in 200 ppm sodium hypochlorite for 2 minutes	f
	Immersion[b] in 200 ppm of sodium hypochlorite for 15 minutes followed by immersion[b] in coconut soap by 1 minute and immersion[b] in 200 ppm sodium hypochlorite for 2 minutes and rinsing[c]	g
Industry 3	Washing followed by immersion[b] in 200 ppm of sodium hypochlorite sanitizer for 15 minutes	h
	Washing followed by immersion[b] in 200 ppm of sodium hypochlorite sanitizer for 15 minutes and rinsing[c]	i
Industry 4	Washing followed by immersion[b] in 200 ppm of sodium hypochlorite sanitizer for 1 minute	j
	Washing followed by immersion[b] in 200 ppm of sodium hypochlorite sanitizer for 1 minute and rinsing[c]	k
Industry 5	Washing followed by immersion[b] in 200 ppm of sodium hypochlorite sanitizer for 5 minutes	l
	Washing followed by immersion[b] in 200 ppm of sodium hypochlorite sanitizer for 5 minutes and rinsing[c]	m

[a, c] Performed with 250 mL of potable water at 22 ºC; b Immersion in 500 mL of 200 ppm of free chlorine solution at 22 ºC.

0.1% peptone water and plated (20 µL), in triplicate, by the droplet-method on XLD (HiMedia, Mumbai, India) agar plates [16]. The plates were incubated at 37°C for 24 hours.

Statistical analyses were performed with SPSS Statistics version 21 at $p < 0.050$.

Results

Initial microbiological loads showed average counts of 5.83±0.83 log CFU/g. Of the different sanitizing procedures applied by the five industries studied, the majority of companies (four) carried out the washing procedure with potable water as first step of the process. Subsequently, disinfection was carried out using 200 ppm sodium hypochlorite, even though different immersion times were used, depending on the company (1, 2, 5, and 15 minutes, Table 1).

The washing step alone decreased significantly the SE86 population on lettuce, being the reduction approximately 1 log CFU/g (Student's T-test, p = 0.004) (Table 2).

The disinfection performed using 200 ppm sodium hypochlorite was able to reduce 1 to 3 log CFU/g of SE86, depending on the immersion time used (Table 2).

The most effective method has been demonstrated by Industry 3, which used washing followed by disinfection with immersion 200 ppm sodium hypochlorite for 15 minutes and final rinsing. However, no statistical differences were observed among the microbial reductions after immersion times of 1, 2, 5, and 15 minutes, performed by Industries 1, 3, 4, and 5, respectively (Student's T-test, p > 0.005). The procedure adopted by Industry 2 showed the lowest microbial reduction on this step (1.21±0.90 log CFU/g), being this difference significantly lower in comparison to the Industries (Student's T-test, p < 0.001 for all) (Table 2).

Rinsing with potable water was the last procedure and was adopted by all industries. The rinse step provided reductions between 0.12±0.63 log CFU/g to 1.90±1.07 log CFU/g (Table 2).

After the whole procedure (washing + disinfection + rinsing), Industry 5 showed the lowest reduction in SE86 population (4.42 log CFU/g), due to immersing lettuce directly in 200 ppm sodium hypochlorite for 5 minutes. Industry 2 and industry 1 showed similar reductions, *i.e.* 4.48 and 4.49 log CFU/g, respectively. Industry 4 reduced 5.11 log CFU/g and the industry that showed the highest reduction in SE86 population was Industry 3, which used 15 minutes of immersion in 200 ppm sodium hypochlorite followed by rinsing, corresponding to 5.83 log CFU/g. However, considering only the reductions obtained in the disinfection step, no statistical differences were

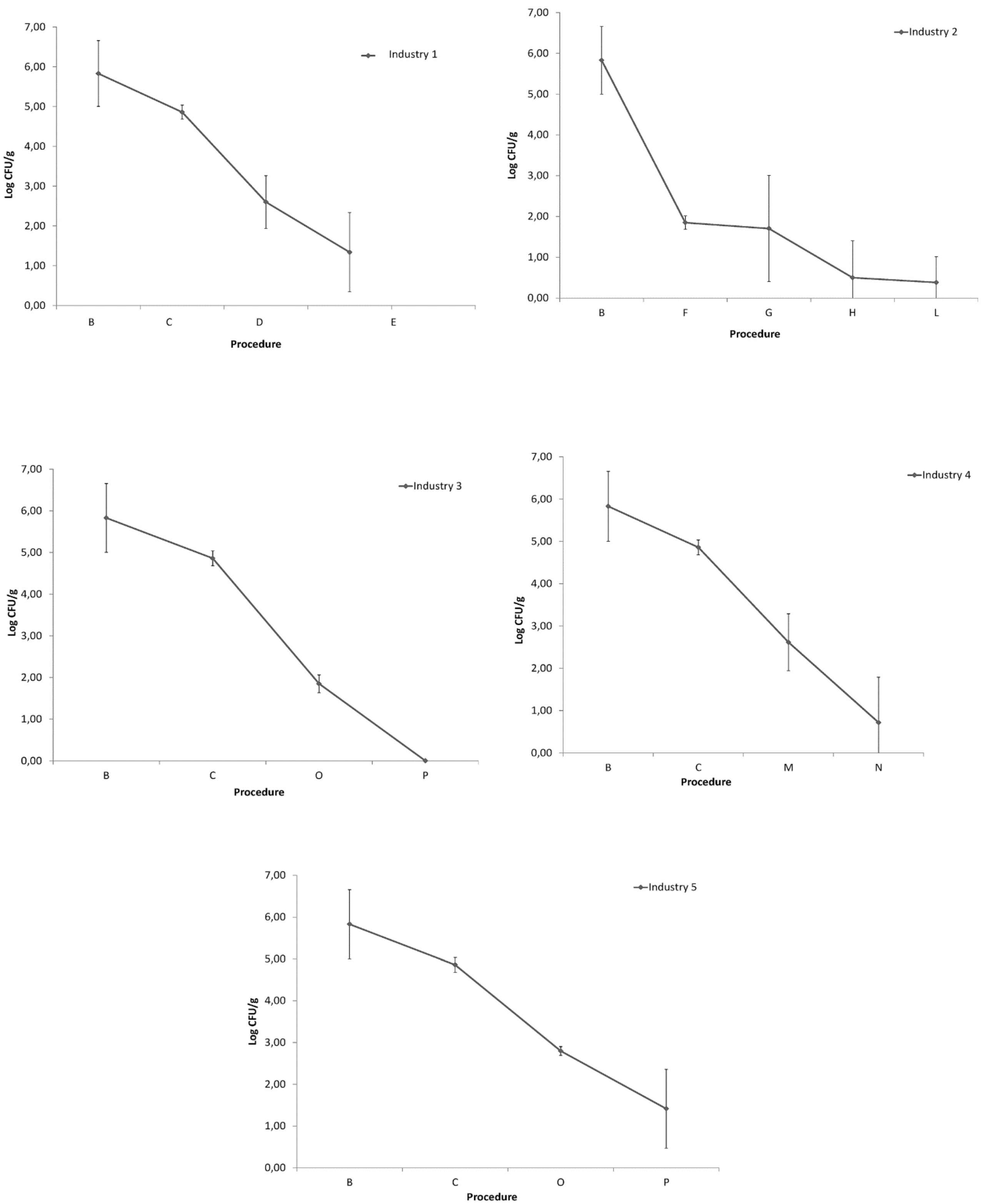
Table 2. *Salmonella* Enteritidis SE86 reduction due to different washing and disinfection procedures observed in minimally processed vegetable industries of Southern Brazil.

Industry	Code	Procedure	SE86 load reduction (log UFC/mL)	Std. Deviation
1	a	Washing [a]	0.97	0.18
	b	Immersion[b] in 200 ppm of sodium hypochlorite sanitizer for 2 minutes	2.26	0.66
	c	Rinsing[c]	1.26	0.99
		Total reduction	4.49	0.67
2	d	Immersion 200 ppm of sodium hypochlorite sanitizer for 15 minutes	3.01	0.16
	e	Immersion in 10% coconut soap by 1 minute	0.14	1.30
	f	Immersion in 200 ppm sodium hypochlorite for 2 minutes	1.21	0.90
	g	Rinsing[c]	0.12	0.63
		Total reduction	4.48	1.35
3	a	Washing [a]	0.97	0.18
	h	Immersion in 200 ppm of sodium hypochlorite sanitizer for 15 minutes	3.01	0.21
	i	Rinsing[c]	1.85	0.00
		Total reduction	5.83	0.82
4	a	Washing [a]	0.97	0.18
	j	Immersion in 200 ppm of sodium hypochlorite sanitizer for 1 minute	2.24	0.67
	k	Rinsing[c]	1.90	1.07
		Total reduction	5.11	0.82
5	a	Washing [a]	0.97	0.18
	l	Immersion in 200 ppm of sodium hypochlorite sanitizer for 5 minutes	2.06	0.10
	m	Rinsing[c]	1.38	0.94
		Total reduction	4.41	0.48

[a, c] Performed with 250 mL of potable water at 22 ºC; b Immersion in 500 mL of 200 ppm of free chlorine solution at 22 ºC.

observed among the procedures adopted by the five industries investigated (Student's T-test, $p > 0.005$ for all) (Figure 1).

Figure 1. Kinetics of *Salmonella* Enteritidis SE86 reduction due to different washing and disinfection procedures observed in minimally processing vegetable industries of Southern Brazil.



Discussion

Minimally processed fresh fruit and vegetables are commonly defined as any fruit and vegetable that has been subjected to different processing steps, *i.e.* peeling, trimming, cutting, washing, disinfection, rinsing [12]. The objective is to obtain a fully edible product, while providing convenience and functionality to consumers and ensuring food safety [17]. Different methods of cleaning and disinfection are adopted by vegetable industries, and, all of these, aim at reducing the organic matter and eliminating pathogens in order to produce safe products.

Inside industries, initial washing should be done and can be achieved very simply by spraying with potable water or immersion of products in chilled water (1-10 °C) [17]. According to the results of the present study, the majority of industries carried out the washing step, and this procedure was responsible for a reduction of approximately 90% (1 log) of SE86 contamination (Table 2). Beuchat *et al.* (2001) and Van Haute *et al.* (2013) also showed that the washing step reduces around 1 log CFU/g of the microbial loads present on lettuces [18,19]. Only Industry 2 did not perform the initial washing with potable water, and, instead of that, this Industry started the process directly with immersion in 200 ppm sodium hypochlorite for 15 minutes, followed by using coconut soap for 1 minute. This procedure was not verified at any other industry, scientific literature or official regulation. Furthermore, the professional responsible for the process was unable to justify the use of this procedure, so this processing was considered inadequate.

In addition to microbial reduction, the initial washing also contributes to the reduction of organic matter naturally present on the vegetables surface. It has been demonstrated that the increase of organic matter in the washing solution has a negative effect on the efficiency of disinfection, once the chlorine sanitizers reacts with organic matter, lowering the disinfecting action [20-22] and also forming trihalomethanes (THMs), which have harmful effects on human health [23]. Van Haute *et al.* (2013) found that higher organic loads lead to a rapid consumption of chlorine, reducing the effectiveness of chlorine disinfection [19]. In fact, the negative aspects of the use of chlorine compounds have induced some European countries, including Germany, Netherlands, Switzerland, and Belgium to prohibit the use of chlorine [24].

Luo *et al*. (2014) have examined cross-contamination prevention during produce washing and specify that free chlorine concentration (*i.e.* disinfectant residual) in the washing water is a main critical control factor for cross-contamination prevention [25,26]. During washing produce, an increasing organic load is evident from the increased chemical oxygen demand (COD) and turbidity in the washing water, and declining disinfectant residual, which can be indirectly estimated by the oxidation reduction potential [27,28]. Briefly, the disinfectant residual and, if relevant, the pH of the washing process water are important to be monitored in industrial tanks.

The efficacy of sanitizers and other interventions aimed at reducing pathogens at acceptable levels has been widely considered [11]. In addition, the goal of disinfection is to prevent the transfer of microorganisms from process water to produce and from a contaminated produce to another produce over time [29]. Typical industrial application of free chlorine concentrations ranges from 50 to 200 mg/L, with a short contact time (*i.e.* 1–2 min), and pH values between 6.0 and 7.5 in order to stabilize the HOCl form alongside minimizing corrosion of processing equipment [23, 28, 30-33]. Maintaining a stable HOCl form during washing remains a challenge since soil, debris, and exudates can accumulate and contribute to an increasing organic load [23,30,22].

In Brazil there is no specific legislation for the use of sanitizing to disinfect fresh-cut vegetables in minimally processed industries, neither regarding the contact time and concentrations. Typically, the minimally processed vegetable industries follow the recommendation from the regulation of Good Manufacturing Practices for food services, which considers the following steps: a) washing using potable water, b) disinfection by immersion in 200 ppm of free chlorine solution, for 15 minutes, and c) rinsing with potable water [34].

As it can be seen in Table 2, the reduction of SE86 population in the disinfection process ranges from 1 to 3 log CFU/g. Corroborating these results, other studies have shown that chlorine solution significantly reduces the microbial load of minimally processed vegetables. The reduction observed in other studies ranged from 1 log CFU/g to 3.15 log CFU/g, depending on the inoculation method used in tests, concentration, contact time, and initial population of microorganisms [35-39,4].

The last procedure adopted by all industries was rinsing. The rinse step is recommended by the Codex Alimentarius [13]. Besides this purpose, reductions were demonstrated by rinse, 0.12±0.63 log CFU/g to 1.90±1.07 log CFU/g.

The most effective method showed by this study was the use of washing followed by 15 minutes of immersion in 200 ppm sodium hypochlorite and, then, rinsing with potable water, which reached a reduction of 5.83 log CFU/g in SE86 population (Figure 1). This method is the one recommended by a Brazilian regulation of Good Manufacturing Practices for Food Services of RS [34]. However, no statistical differences were observed in relation to the reduction rates obtained by other disinfection methods in industries investigated. Thus, all disinfection procedures applied by the industries investigated showed the same effectiveness to reduce SE86 population on lettuce.

It has been demonstrated that *Salmonella* can grow until 8 log CFU/g on lettuce leaves and chemical treatment cannot ensure the complete destruction of microbial contaminants on the vegetable surface [29,12]. So, the washing and disinfection methods should reduce microbial counts until safety levels.

Fresh-cut produce must be managed in primary production phases and be produced for marketing following strict control procedures in order to reduce overall quality loss and assuring its safety to consumers [30]. In this research, besides Industry 2, the average reduction rate in the disinfection steps did not differ significantly among the industries. Thus, the minimally processed vegetable industries investigated showed a very similar reduction capability, differing only in the time of immersion in disinfection solution (1 to 15 minutes).

Conclusion

Regarding the different washing and disinfection procedures observed in minimally processed vegetable industries investigated, the most effective method was the use of initial washing, followed by 15 minutes of immersion in 200 ppm sodium hypochlorite and then rinsing with potable water. However, immersion times of 1, 2, and 5 minutes in 200 ppm sodium hypochlorite showed similar reduction rates for *Salmonella* Enteritidis SE86 CFU, indicating that industries could adopt shorter disinfection times (when comparing to 15 minutes) in order to reduce process costs and also significantly reduce the numbers of *Salmonella spp.* on lettuce leaves.

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