FOODBORNE PATHOGENS AND DISEASE
Volume 13, Number 8, 2016

DOI: 10.1089/fpd.2015.2079

Preliminary Study on the Effect of Fermented Cheese Whey on *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* Goldcoast Populations Inoculated onto Fresh Organic Lettuce

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Abstract

Cheese whey fermented by an industrial starter consortium of lactic acid bacteria was evaluated for its antibacterial capacity to control a selection of pathogenic bacteria. For their relevance on outbreak reports related to vegetable consumption, this selection included *Listeria monocytogenes*, serotype 4b, *Escherichia coli* O157:H7, and *Salmonella* Goldcoast. Organically grown lettuce was inoculated with an inoculum level of $\sim 10^7$ colony-forming unit (CFU)/mL and was left for about 1 h in a safety cabinet before washing with a perceptual solution of 75:25 (v/v) fermented whey in water, for 1 and 10 min. Cells of pathogens recovered were then counted and their number compared with that obtained for a similar treatment, but using a chlorine solution at 110 ppm. Results show that both treatments, either with chlorine or fermented whey, were able to significantly reduce ($p < 0.05$) the number of bacteria, in a range of 1.15–2.00 and 1.59–2.34 CFU/g, respectively, regarding the bacteria tested. Results suggest that the use of fermented whey may be as effective as the solution of chlorine used in industrial processes in reducing the pathogens under study (best efficacy shown for *Salmonella*), with the advantage of avoiding health risks arising from the formation of carcinogenic toxic chlorine derivates.

Introduction

Due to interest for healthier diets, the demand of fruits and vegetables, whole or minimally processed (MP), has been increasing over the last few years (OMAIAA, 2011; Olaimat and Holley, 2012). However, the number of outbreaks and cases of foodborne illnesses associated to the consumption of leafy vegetables has increased in the last few years, and consequently, the concern about the presence of pathogens in these foodstuffs (Sagong *at al*., 2011; Painter *et al.*, 2013).

In fact, the contamination of vegetables reflects the microbiota of the environment where they are cultivated (Tauxe *et al.*, 1997; Holden *et al.*, 2009). In addition, mishandling of vegetables during harvest and postharvest, transportation, processing, and packaging can also turn these products into vehicles of pathogenic microorganisms (Heard, 2002). Data from the United States, in the period between 1998 and 2008, reported that fruits and vegetables accounted for 46% of the foodborne diseases (most often caused by norovirus, *Salmonella* spp., and *Escherichia coli* O157:H7), with leafy vegetables belonging to the largest numbers (Painter *et al.*, 2013). Europe reported, from 2008 to 2013, 215 outbreaks of food and waterborne diseases, 63% related to salmonellosis, with 31% linked to vegetables. The great German outbreak with 50 deaths caused by *E. coli* O104:H4 is within the last category (WHO, 2011; Gossner *et al.*, 2015). *Salmonella* spp., *E. coli* O157:H7, and *Listeria monocytogenes* are the pathogens of major concern implicated in numerous outbreaks of foodborne illnesses associated to the consumption of leafy vegetables, according to Sagong *et al.* (2011).

Washing with sanitizers is normally the only step used in MP leafy vegetables, to assure the reduction in the number of pathogens and spoilage microorganisms. Chlorine has been the sanitizer more often used in industry, to ensure the safety of this food product. Meanwhile, concern about the

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environmental and consumers' health risk due to the formation of chlorine carcinogenic toxic derivatives, like trihalomethanes and chloramines, has led to increased research on new methodologies that simultaneously reduce pathogens and toxic chemicals (Sapers, 2003; Martin-Diana *et al.*, 2006; Martinez-Sanchez *et al.*, 2006; Gil *et al.*, 2009; Ölmez and Kretzschmar, 2009; Francis *et al.*, 2012). Our previous work (Santos *et al.*, 2015) used naturally fermented cheese whey, containing industrial lactic acid starter bacteria, as a sanitizer with promising results on hygiene indicator microorganism reduction. The present work was performed to evaluate if that antibacterial effect demonstrated for fermented cheese whey has the same magnitude of inhibition over the three major pathogens reported in outbreaks and linked to consumption of raw vegetables. This study was conducted to assess if the contact time of 1 and 10 min of fermented cheese whey and sodium hypochlorite solutions are equally efficient as sanitizers against these three pathogens spiked onto lettuce leaves.

Materials and Methods

Bacterial strains and culture preparation

Three bacterial species, found in higher frequency on raw fruits and vegetables and reported on outbreaks (SCF, 2002), were selected to be used in this work following the recommendations of the European Standard EN 1276:2009. *L. monocytogenes* NCTC 11994, serotype 4b, *Salmonella* Goldcoast NCTC 13175, and *E. coli* O157:H7 NCTC 12900 were provided by the Food Microbiology Laboratory, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisbon, Portugal. Inocula were prepared from stock cultures stored at −80°C, transferred into 10 mL of brain-heart Infusion (Oxoid™, Hampshire, United Kingdom) for two consecutive cultures with 24 h intervals, struck afterward on Tryptone Soya Agar with Yeast Extract (TSYEA; bioMérieux® SA, Marcy l'Étoile, France), and incubated at 37°C ± 1°C overnight. A suspension of these cultures was made in Maximum Recovery Diluent (MRD; bioMérieux SA), corresponding to $\sim 10^7$ colony-forming unit (CFU)/mL. Bacterial populations in these suspensions were determined by surface plating duplicate samples on Compass® *Listeria* Agar, Compass *Salmonella* Agar, and CT-SMAC (all Biokar Diagnostics, Beauvais, France) for *L. monocytogenes*, *Salmonella* Goldcoast, and *E. coli* O157:H7, respectively. After serial dilution in MRD, the plates were incubated at 37°C ± 1°C for 24 h, after which colony counts were recorded.

Sample inoculation

Lettuce (*Lactuca sativa* var. *crispa*), manure fertilized grade, was purchased from a certified organic grower, at a local market. Two or three damaged outer lettuce leaves were discarded in all samples and the unspoiled ones were cut, using a metal cutter with 6 cm diameter. Samples of leaves (50 g) were spiked by immersion in 500 mL of MRD containing individually the suspensions of bacteria described above, for 1 min. Afterward, each inoculum was decanted and the lettuce placed separately on a sterile perforated tray to drain and left to dry in a biosafety cabinet at room temperature (22°C ± 4°C) for about 1 h.

Preparation of washing solutions

Whey, obtained as waste from a cheese factory, fermented as previously described (Santos *et al.*, 2015) has, undiluted, a batch average of (g/L): lactic acid 18, acetic acid 0.89, ethanol 7.46, and a final pH of 3.19. A percentage solution of fermented whey in water (75:25, v/v) and a chlorine solution (110 ppm) were prepared as previously used (Santos *et al.*, 2015).

Washing treatments

Lettuce leaves (10 g), spiked with each one of the bacterial species, were placed into a Stomacher bag (PE; Seward Ltd., London, United Kingdom) either with: (1) no sanitizer solutions (control), (2) 50 mL of 110 ppm sodium hypochlorite or (3) 50 mL of diluted fermented whey for 1 and 10 min of contact time. All samples were shaken at 4°C using an incubator with orbital shaking (Panasonic MIR 154, Tokyo, Japan).

We consider the initial bacterial load that was attached onto lettuce leaves, as determined for treatment of (1) our control reference, because previous studies revealed that washing with water reduced 0.5–1.0 log CFU/g (Singh *et al.,* 2002; Sagong *et al.*, 2011; Santos *et al.,* 2015).

Microbiological analyses

After the washing step, leaves were transferred into new sterile Stomacher bags, diluted 1:10 with Buffered Peptone Water (bioMérieux SA) and then homogenized in the Stomacher (Stomacher 400 Circulator; Seward Limited, London, United Kingdom) for 1 min at 230 rpm for bacterial evaluations. Serial dilutions of the initial suspensions were made in MRD and then surface plated in duplicate (0.1 mL) onto Compass *Listeria* Agar, Compass *Salmonella* Agar, and CT-SMAC, with incubation at 37°C ± 1°C for 24 ± 2 h. To assure that only the spiked bacteria were recovered and compared with the initial load, representative colonies were picked up to TSYEA for confirmation and, subsequently, inoculated into API *Listeria* (bioMérieux SA), Triple Sugar Iron, and Indole test (both Biogerm, Maia, Portugal), for *L. monocytogenes*, *Salmonella* Goldcoast, and *E. coli* O157:H7, respectively, for biochemical identification.

Statistical analysis

Counts were statistically analyzed using the software SigmaPlot (version 12.5) from StatSoft (Tulsa, OK), to perform a two-way analysis of variance with a Tukey's test to compare differences between groups at $p < 0.05$.

Results and Discussion

Fresh organic lettuce leaves inoculated with *L. monocytogenes*, serotype 4b, *E. coli* O157:H7, and *Salmonella* Goldcoast were used to assess the effect of fermented cheese whey on the reduction of bacterial cell numbers. Immediately after inoculation, lettuce samples used as control, presented initial populations of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* Goldcoast of 6.34, 5.61, and 5.99 log CFU/g, respectively. The reductions obtained after 1 and 10 min contact time with the sanitizer solutions assayed are presented in Table 1. As a general note, results show that both

Table 1. Populations of *Listeria monocytogenes, Escherichia coli* O157:H7, and *Salmonella* Goldcoast Recovered from Lettuce Leaves Following Treatments

Assays		*Strains*					
		L. monocytogenes		E. coli *O157:H7*		Salmonella *Goldcoast*	
Contact time (min)	*Treatments*	*Population recovered (log CFU/g)*	*Reduction (log CFU/g[1])*	*Population recovered (log CFU/g)*	*Reduction (log CFU/g)*	*Population recovered (log CFU/g)*	*Reduction (log CFU/g)*
1	No sanitizer—initial cell load	6.34 ± 0.20^{a}	—	5.61 ± 0.09^{a}	—	5.99 ± 0.06^{a}	—
	Chlorine solution (100 ppm)	4.22 ± 0.10^{b}	1.59 ± 0.14	2.68 ± 0.10^{b}	2.20 ± 0.02	3.34 ± 0.07^{b}	1.99 ± 0.13
	Fermented whey (75:25 v/v)	4.80 ± 0.09^{c}	1.15 ± 0.20	2.97 ± 0.06^{b}	1.98 ± 0.04	4.11 ± 0.14^{d}	1.41 ± 0.11
10	No sanitizer—initial cell load	6.34 ± 0.20^{a}	—	5.61 ± 0.09^{a}	—	5.99 ± 0.06^{a}	—
	Chlorine solution (100 ppm)	3.91 ± 0.33^{b}	1.77 ± 0.16	2.80 ± 0.72^{b}	2.12 ± 0.64	2.86 ± 0.14^{c}	2.34 ± 0.19
	Fermented whey (75:25 v/v)	4.62 ± 0.36^{c}	1.23 ± 0.44	3.11 ± 0.57^{b}	1.89 ± 0.49	3.32 ± 0.24^{e}	2.00 ± 0.25

Values are mean ± standard deviation of duplicates of three independent trials. Different letters represent significant differences between values ($p < 0.05$).
CFU, colony-forming unit.

chlorine and fermented whey treatments were able to significantly reduce ($p < 0.05$) the level of all bacteria tested, compared to the numbers of bacterial cells present in the controls. However, the efficacy of the chlorine and whey treatments varied with the characteristics of the bacterial species under test.

With respect to the contact times studied, 1 and 10 min with chlorine or whey as sanitizers, the results show that no significant reduction ($p < 0.05$) was observed on numbers of *L. monocytogenes* by increasing the length of time. Gram-positive bacterial cells are more permeable to molecules in general, due to their content in peptidoglycan and lipopolysaccharide of membranes.

However, for each contact time, significant differences were found in *L. monocytogenes* cell numbers between both sanitizers and the control, with chlorine producing slightly better results. Chlorine is forbidden as sanitizer of MP vegetables, in Germany, The Netherlands, Switzerland, and Belgium, for consumer health risk, due to formation of toxic compounds (Rico *et al.*, 2007).

The results obtained for reduction in numbers of log CFU/g of *Salmonella* Goldcoast were essentially identical on the capacity of chlorine and fermented whey to remove *Salmonella* Goldcoast and *L. monocytogenes* cells from lettuce leaves. A significant difference in number reductions between both treatments, compared to the initial cell load used ($p < 0.05$), showed that chlorine was able to induce a higher reduction in bacterial counts and that the time of contact of both sanitizers is relevant.

Despite the fact that *E. coli* and *Salmonella* are both Gram-negative pathogens and belong to the same *Enterobacteriaceae* family, the effect of the sanitizers tested and the two times assayed as treatments produced different results.

Overall, it is possible to conclude that the duration of treatments (1 or 10 min) did not produce a dramatic difference in cell count numbers for the three bacterial species under analysis. Nevertheless, small differences were encountered, with longer contact times of the inoculated lettuce leaves with the sanitizers (i.e., 10 min) being more efficient in reducing bacterial cell numbers in the cases of *L. monocytogenes* and *Salmonella* Goldcoast and smaller (i.e., 1 min) contact times originating better results in the case of *E. coli* O157:H7.

In a treatment with lactic acid at 2% (w/v; a concentration higher than that used in the present study; the whey used in our experiments contained 1.4% w/v lactic acid), Sagong *et al.* (2011) found a reduction of 1.74 ± 0.38, 1.73 ± 0.16, and 1.30 ± 0.11 for *E. coli* O157:H7, *Salmonella* Typhimurium, and *L. monocytogenes*, respectively, very similar to the reductions in numbers of log CFU/g we found in this work for equivalent bacterial species.

Many works have been published with alternative methods for vegetable disinfection, such as deionizer water (Beuchat, 1998; Singh *et al.*, 2002; Rodgers *et al.*, 2004; Ölmez and Akbas, 2009), hydrogen peroxide (Lin *et al.*, 2000; Samadi *et al.*, 2009), organic acids (Beuchat *et al.*, 2004; Bari, *et al.*, 2005; Martinez-Sanchez *et al.*, 2006; Zhang *et al.*, 2009), and irradiation (Minter and Foley, 2006; Sagong *et al.*, 2011). As far as we are aware, solutions of whey fermented by lactic acid bacteria (LAB) have never been assayed before. LAB have been used traditionally for food preservation in dairy and meat products, as well as in fermented vegetables. The antimicrobial activity exhibited by LAB fermented whey may be explained by a set of characteristics, such as production of organic acids, mainly lactic acid, bioactive peptides, and hydrogen peroxide (Trias *et al.*, 2008).

In conclusion, we can say that fermented cheese whey solution has achieved a significant reduction of the lettuce leaf-inoculated population of the three pathogenic bacteria studied, with results that closely approximate those obtained with the chemical sanitizer chlorine. Fermented whey is, therefore, a promising natural product that can be a good alternative to the use of chlorine in fresh vegetable disinfection. On a previous work, no color or change on general aspect of leaves was recorded (Santos *et al.*, 2015). Because no extra washing is needed after treatments, one may add that fermented whey contains about 10^6 CFU/g live natural LAB (Santos *et al.*, 2015), known to have a beneficial modulating effect on the gut microbiota and to favor human health (Prakash *et al.*, 2011).

This was a preliminary study with important outcomes, which open novel cost-effective and health-promoting perspectives in the disinfection of MP salads, using fermented

whey, but being a preliminary result, further work should be done to test additional strains of each bacteria used, as well as other pathogenic bacteria and different exposure times.

Acknowledgments

The authors acknowledge the following institutions: Food Microbiology Laboratory of Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisbon, Portugal, for providing the strains used in this work; and Sara Bernardes Silva, Technical Director of Indústria de Laticínios SA, Portugal, for kindly providing the whey used in this work.

Disclosure Statement

No competing financial interests exist.

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