Fresh produce and microbial contamination: persistence during the shelf life and efficacy of domestic washing methods

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Abstract
The transmission of enteric pathogens by fresh produce depends on the survival of the bacteria organisms during the product shelf-life. The removal of any potentially hazardous microorganism from the vegetables is therefore dependent on the washing and sanitizing techniques employed by individual households. For this purpose, in this work we investigated the persistence of enteric bacteria, using as model *Salmonella enterica* serovar Napoli (*S.* Napoli) and *Yersinia enterocolitica*, in vegetables stored at refrigeration temperature (4 °C). The efficiency of tap water and different chlorine solutions for cleaning vegetables experimentally contaminated with *Y. enterocolitica* were tested. The results showed that in lettuce spiked with different concentrations of *S.* Napoli and *Y. enterocolitica*, both microorganisms were still detected after seven days of storage at 4 °C. Lettuce contaminated with low concentrations of *Y. enterocolitica* was not decontaminated by washing with tap water or with water added with 60 ppm of chlorine. The presence of *Y. enterocolitica* in lettuce was reduced of about 1-2 logs after washing with water added with 220 ppm of chlorine. The addition of low concentration of chlorine in post harvest washing processes represents a useful tool to reduce the contamination of the vegetables, with consequent reduction of the risks. However, since complete decontamination was not achieved, foodborne infections linked to fresh produce can still be possible, although contamination is avoided during primary production.

INTRODUCTION

The role of fresh fruits and vegetables in a healthy diet is well recognized in nutrition researches as they ensure an adequate intake of vitamins, minerals, fibres and antioxidants. Bacterial pathogens have been sometimes associated with fresh produces, especially with leafy vegetables. The increasing demand of minimally processed foods and the absence of any thermal inactivation process have led to consider these products as a serious safety concern for governments and industry. The consumption of fresh-cut vegetables has been associated with several food-borne outbreaks all over the world [1]. From 1990 to 2005, in the United States about the 13% of reported food-borne outbreaks was linked to fresh produce [2]. The number of reported outbreaks, associated with food of non-animal origin (FNAO) including leafy greens has amplified at European level [3], where accounted for 6.6% of all strong-evidence outbreaks, included vegetables, fruits, cereals, sprouts, herbs and spices and products thereof (4.5%) [4].

Leafy green vegetables, usually grown in natural environment, can be contaminated by bacterial pathogens from fecal origin, that have been identified as the main causative agents in numerous outbreaks linked to this food [5].

In Italy, a large monitoring on the presence enteric pathogens harmful to human health in fresh leafy (FL)

and "fresh-cut" or "ready-to-eat" (RTE) vegetable products, has showed the contamination in 3.7% of 1372 FL vegetable products and 1.8% of 1160 RTE vegetable products retailed in supermarkets or farm markets [6]. Other recent similar studies in different Countries, have demonstrated that bacterial contamination of leafy herbs was frequently found but at a low rate [7].

Contamination of vegetables with pathogenic microorganisms of human health significance can occur directly or indirectly via animals or insects, soil, water, dirty equipment, and human handling [8]. RTE vegetables collected from three Italian production systems have showed that the presence of pathogenic bacteria was closely related to the different agricultural and hygiene practices involved in their production [2].

The transmission of enteric pathogens by fresh produce dependents on the survival of the pathogens during the periods of the shelf-life, that in Europe ranges between 5 and 7 days. *Salmonella* outbreaks constitute a significant portion of all fresh produce related outbreaks and this bacterium has been isolated from a broad range of vegetables [6]. Among the *Salmonella* serovars, *S.* Napoli ranks fourth accounting for 5% of all serovars isolated from human infection in Italy [9]. *S.* Napoli has been isolated from vegetables, and the consumption of fresh leafy vegetables (e.g. rocket salad grown in Italy) has been linked to outbreaks of *S.* Napoli [10]. However, specific risk factors for *S.* Napoli infection were not identified so far, and the existence of environmental *S.* Napoli reservoir has been proposed [10]. Pathogenic *Y. enterocolitica* has been occasionally detected in vegetables and surface water; thus vegetables and untreated water could be potential sources for human yersiniosis. *Y. enterocolitica* rivals with *Salmonella* as a foodborne pathogen in fresh products, because it is a psychotropic zoonotic pathogen. The ability of *Y. enterocolitica* to grow at refrigeration temperature increases the concern in terms of food safety on its presence in refrigerated food, like RTE vegetables [11].

Household washing, used to eliminate field dirt and debris in FL and RTE vegetables, also decreases their microbial contamination. The removal of any potentially hazardous microorganism from the vegetables is therefore dependent on the washing and sanitizing techniques employed by individual households [12, 13]. Household washing can be simply done with tap water or added with different antimicrobial agents. Chlorination of the washing water is considered to be one of the best ways to minimize the transmission of pathogens and is the most commonly used sanitizer to treat fresh products, due to its efficacy, low cost, and simple use.

Water solutions usually contain concentrations of 50-200 ppm available chlorine [14].

In this context, the objectives of this work were to investigate on:

• the persistence of enteric bacteria, using as model *S.* Napoli and/or *Y. enterocolitica*, in vegetables stored at refrigeration temperature (4 °C);

• the efficiency of tap water, chlorine solution at 50 and 220 ppm to clean experimentally contaminated samples by using vegetables inoculated with *Y. enterocolitica* as model.

MATERIALS AND METHODS

Culture media

Buffered peptone water (BPW), Rappaport-Vassiliadis soja broth (RVS), Muller-Kauffmann Tetrathionate-novobiocin broth (MKTTn), Xylose-Lysine-Desoxycholate Agar (XLD), Brilliant Green Agar (BGA), Triple Sugar Iron Agar (TSI), Tryptone Soy Broth (TSB) and Nutrient Agar, were from Oxoid Ltd, Basingstoke, United Kingstone. Yersinia PSB broth (PSB) and Cin Agar Base (CIN) were from Biolife Italiana S.r.l., Milano, Italy.

Bacteria strains

Isolates of *Salmonella* Napoli and *Y. enterocolitica* O:3 biotype 4, were supplied by the Pathogenic Enterobacteria Unit of the Istituto Superiore di Sanità (Rome, Italy).

Y. enterocolitica was cultured in TSB and incubated for 48 h at 30 °C while *Salmonella* incubated in TSB for 24 h at 37 °C.

Persistence of Salmonella spp and Yersinia enterocolitica on the surface of lettuce

1250 g of lettuce was divided into fifty aliquots of 25 g each, and spiked respectively:

a) eight aliquots with 1 ml of a suspension 10^5 CFU/ml of *Y. enterocolitica*;

b) eight aliquots with 1 ml of a suspension 10^3 CFU/ml of *Y. enterocolitica*;

c) eight aliquots with 1 ml of a suspension 10^1 CFU/ml of *Y. enterocolitica*;

d) eight aliquots with 1 ml of a suspension 10^5 CFU/ml of *S.* Napoli;

e) eight aliquots with 1 ml of a suspension 10^3 CFU/ml of *S.* Napoli;

f) eight aliquots with 1 ml of a suspension 10^1 CFU/ml of *S.* Napoli.

The last two aliquots were incubated in 225 ml of BPW for 20 h at 37 °C and 225 ml of PSB for 8 h at 25 °C, to verify the absence of *Salmonella spp* and *Y. enterocolitica*, respectively.

The contaminated aliquots were stored at 4 °C. Each day, for a total of seven days (day 1 to day 7), one sample of aliquots a, b, c, d, e and f was collected and incubated respectively and separately in 225 ml of PSB for 48 h at 25 °C (aliquots a, b and c) and in 225 ml BPW for 20 h at 37 °C (aliquots d, e and f).

The eighth sample (day 0) of all contaminated aliquots was immediately incubated in 225 ml of PSB for 48 h at 25 °C (aliquots a, b and c) and in 225 ml BPW for 20 h at 37 °C (aliquots d, e and f). In *Figure 1* was reported the scheme about the experimentally contamination.

All the aliquots were analysed by ISO methods (ISO 6579 for *S.* Napoli and ISO 10273 for *Y. enterocolitica*) and Real-Time PCR (RT-PCR) methods according to Delibato *et al.* [15] and Made *et al.* [16]. Cycle threshold values of each experiments were recorded for statistical analysis.

Evaluation of sanitization processes efficiency

Five aliquots of 300 g of Romaine lettuce (*Lactuca sativa* L. var. *longifolia*) were collected and immersed
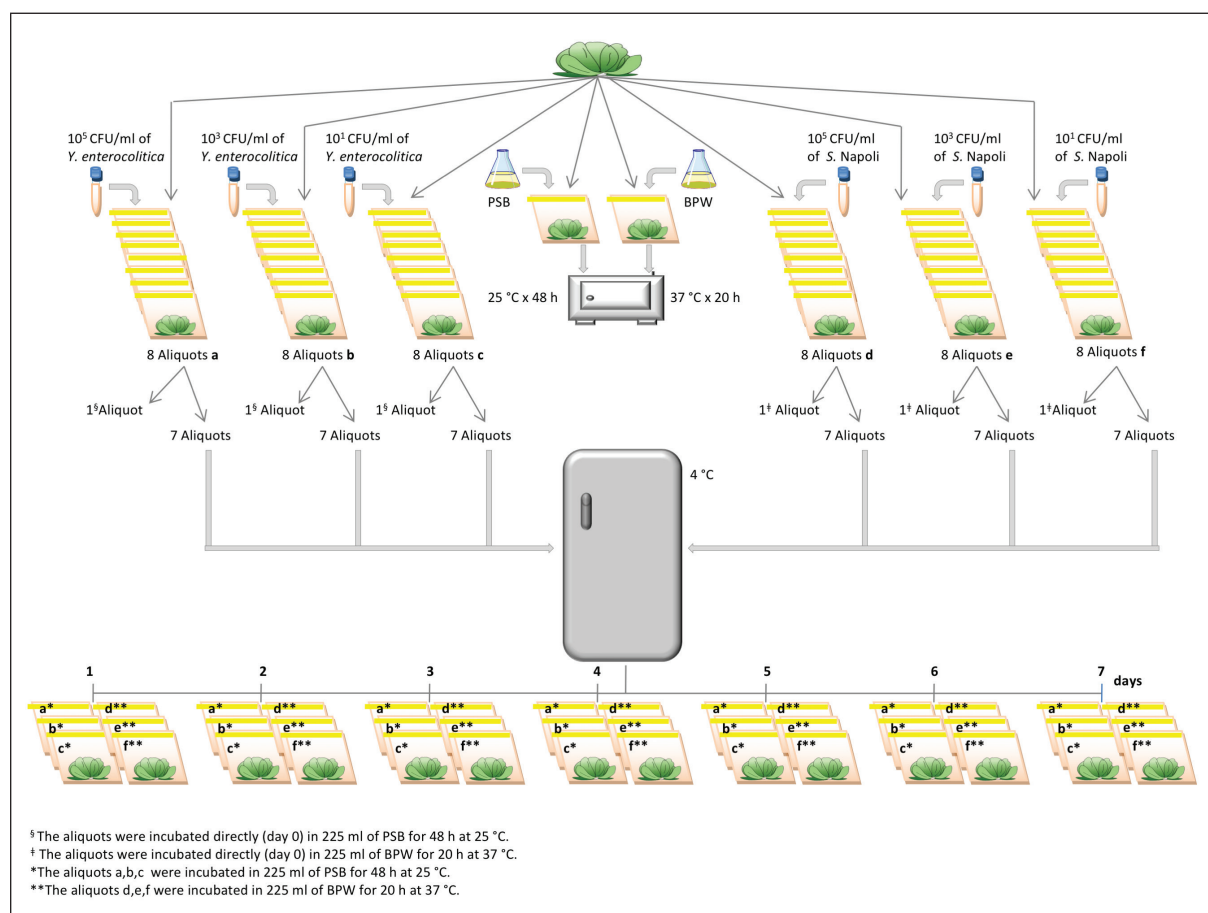
Figure 1

Scheme of experimentally contamination of *Salmonella* or *Yersinia enterocolitica* on the surface of vegetables stored at 4 °C.

in five tanks containing 1800 ml of water for 30 min, strained and left out to dry for 20 min, according with Croci *et al*. [17]. The water in each tank was added with approximately 10^5 or 10^4 or 10^3 or 10^2 or 10^1 CFU/ml of *Y. enterocolitica*. One aliquot of 25 g was initially analyzed to verify that the product was *Y. enterocolitica* free. In order to simulate domestic washing, 25 g of vegetable from each tank was washed with 1000 ml of tap water for approximately 5 min or immersed in 1000 ml of distilled water added with NaClO (for a final concentration of 220 ppm of chlorine) or immersed in 1000 ml of NaClO (for a final concentration of 60 ppm of chlorine). The last two samples were then washed with tap water. The experiments were repeated three times in different days. The samples were incubated in 225 ml of PSB for 48 h at 25 °C.

The presence of pathogenic *Y. enterocolitica* at different concentrations after sanitizing treatments was detected before (T = 0 h) and after the enrichment step (T = 48 h). All samples were analyzed using the standard cultural method and RTi-PCR assay.

Statistical analysis

Cycle threshold (Ct) values of the experiment concerning the evaluation of the effectiveness of different washing procedures were compared using the one-way analysis of variance (ANOVA) with *Tukey post hoc com-*

parison. The tests were undertaken using GraphPad Prism software version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). The null hypothesis was rejected with a *P* value lower than 0.05.

RESULTS

The results, obtained using both, the cultural and molecular assays on lettuce spiked using contaminated water with different concentrations of *S*. Napoli and *Y. enterocolitica,* were reported in *Table 1*. The testing of experimental material, to assess freedom from contamination with *S*. Napoli or *Y. enterocolitica*, showed that uninoculated vegetable samples, tap and distilled water before the addition of chlorine solution where free of the two enteropathogenic bacteria.

For this reason the detection of viable *S*. Napoli or *Y. enterocolitica,* during the storage of lettuce at refrigerated temperature (4 °C) or *Y. enterocolitica* after cleaning, can be entirely ascribed to the experimental inoculation.

The inoculation levels used in this work were higher than natural contamination, but already used in studies published by other Authors [12, 17]. The high concentration of bacteria allows to better evaluate the microorganism behaviors during storage and the differences in the effect of different cleaning procedures.

All the samples, including those contaminated with

Table 1
Persistence of *Salmonella* or *Yersinia enterocolitica* on the surface of vegetables stored at 4 °C

	Salmonella					
	10^5 CFU*		10^3 CFU*		10^1 CFU*	
Time (days)	ISO 6579	RT-PCR (Ct ± SD)	ISO 6579	RT-PCR (Ct ± SD)	ISO 6579	RT-PCR (Ct ± SD)
0	+	22.84 ± 0.12	+	23.70 ± 0.23	+	24.03 ± 0.10
1	+	22.42 ± 0.15	+	23.87 ± 0.15	+	24.85 ± 0.22
2	+	22.87 ± 0.26	+	23.99 ± 0.19	+	25.02 ± 0.12
3	+	23.04 ± 0.25	+	24.12 ± 0.25	+	25.82 ± 0.26
4	+	23.56 ± 0.27	+	24.41 ± 0.23	+	26.35 ± 0.12
5	+	23.89 ± 0.24	+	24.86 ± 0.25	+	25.93 ± 0.21
6	+	23.98 ± 0.26	+	25.00 ± 0.15	+	25.98 ± 0.29
7	+	24.01 ± 0.36	+	24.99 ± 0.21	+	25.89 ± 0.12
	Yersinia enterocolitica					
	10^5 CFU*		10^3 CFU*		10^1 CFU*	
Time (days)	ISO 10273	RT-PCR (Ct ± SD)	ISO 10273	RT-PCR (Ct ± SD)	ISO 10273	RT-PCR (Ct ± SD)
0	+	20.23 ± 0.48	(Ct ± SD)	26.30 ± 0.23	+	29.82 ± 0.23
1	+	21.80 ± 0.26	+	28.96 ± 0.13	+	32.78 ± 0.26
2	+	23.22 ± 0.49	+	29.83 ± 0.24	+	33.06 ± 0.32
3	+	24.82 ± 0.24	+	25.46 ± 0.54	+	31.57 ± 0.18
4	+	20.05 ± 0.27	+	26.84 ± 0.46	+	31.68 ± 0.29
5	+	22.95 ± 0.34	+	28.32 ± 0.48	+	32.09 ± 0.29
6	+	21.69 ± 0.28	+	29.24 ± 0.52	+	33.09 ± 0.23
7	+	22.71 ± 0.31	+	28.22 ± 0.54	+	32.89 ± 0.22

* in 25 g of product; RT-PCR: Real-Time PCR; Ct ± SD: Cycle threshold values ± Standard Deviation.

low concentration of *S.* Napoli or *Y. enterocolitica*, have showed the presence of both microorganisms, after seven days of storage at 4 °C, that is the shelf life limit in Europe for RTE vegetables.

The results obtained by washing the lettuce contaminated with different concentrations of *Y. enterocolitica*, using three different procedures (tap water, distilled water added with sodium hypochlorite at 60 ppm and 220 ppm), are reported in *Table 2*.

The effectiveness of these three procedures has been evaluated for each level of contamination. No statistical difference was found when the three different washing procedures were used to soak lettuce contaminated with distilled water containing high concentration of *Y.*

Table 2
Effectiveness of washing with tap water, tap water added with 220 ppm of sodium hypochlorite and tap water added with 60 ppm of sodium hypochlorite to eliminate *Yersinia enterocolitica* contamination on the surface of vegetables. Assessment at two experimental steps: T = 0 hours and T = 48 h of enrichment (see Methods for details)

Yersinia enterocolitica *	Tap water				Tap water + 220 mg/L of NaClO				Tap water + 60 mg/L of NaClO			
	ISO 10273		RT- PCR (Ct ± SD)		ISO 10273		RT-PCR (Ct ± SD)		ISO 10273		RT-PCR (Ct ± SD)	
Experimental time (hours)	0	48	0	48	0	48	0	48	0	48	0	48
10^5	+	+	> 40	24.06 ± 0.21	-	+	> 40	24.31 ± 0.35	-	+	> 40	24.45 ± 0.24
10^4	-	+	> 40	27.94 ± 0.24	-	+	> 40	28.62 ± 0.65	-	+	> 40	28.31 ± 0.20
10^3	-	+	> 40	27.47 ± 0.28	-	+	> 40	29.25 ± 0.30	-	+	> 40	29.62 ± 0.28
10^2	-	+	> 40	28.85 ± 0.39	-	+	> 40	34.25 ± 0.37	-	+	> 40	28.45 ± 0.31
10^1	-	+	> 40	37.80 ± 0.12	-	-	> 40	No Ct	-	+	> 40	37.82 ± 0.75

*concentration of *Yersinia enterocolitica* in the contaminated water (1800 ml). RT-PCR: Real-Time PCR; Ct ± SD: Cycle threshold values ± Standard Deviation.

enterocolitica (i.e. 10^5 and 10^4 CFU/1800 ml of distilled water) (p > 0.05).

At medium concentration of *Y. enterocolitica* contamination (i.e. 10^3 or 10^2 CFU/1800 ml of distilled water), no significant difference (p > 0.05) was found when the lettuce was soaked with tap water or distilled water added with 60 ppm of chlorine. A significant statistical difference was instead found when the lettuce was soaked with distilled water added with 220 ppm of chlorine, compared with the results obtained with tap water or distilled water added with 60 ppm of chlorine (p < 0.05)

At low concentration of *Y. enterocolitica* (i.e. 10^1 cfu/1800 ml of distilled water), no significant difference was found when the lettuce was soaked with tap water or distilled water added with 60 ppm of chlorine (p > 0.05). *Y. enterocolitica* was not detected in the lettuce contaminated with 10^1 CFU/1800 ml of distilled water when it was rinsed with distilled water added with 220 ppm of chlorine.

The results obtained using RTi-PCR methods were confirmed by the ISO cultural methods.

DISCUSSION AND CONCLUSIONS

The recent knowledge about vegetables, as a vehicle of foodborne illnesses, has led to increase the research on the persistence of different pathogenic microorganisms on leafy green vegetables. In this work we have demonstrated that *S.* Napoli and *Y. enterocolitica* could survive in RTE fresh produce for all their shelf life without any substantial reduction of their microbial loads. Over the last few years, an increasing number of works have been dedicated to reduce the sources of vegetables contamination, during the different steps of their production [18, 19], and using different strategies to safeguard the quality assurance of RTE vegetables [20]. Contamination with microbial pathogens of leafy green vegetables and consequent foodborne illness can occurred during production, harvest, processing, and transporting, as well as in retail and foodservice establishments and in the home kitchen.

Consumers often play a critical role in the prevention of foodborne infections by the use of safe food handling in domestic kitchen [21]. Washing vegetables, prior to their consumption, is the most important component in a multiple hurdle technology to minimize the microbial contamination of fresh products. This work has demonstrated that the domestic washing procedures are unable to eliminate at all the risk correlated with the presence of enteric pathogenic microorganism in lettuce.

Lettuce contaminated with low concentrations of *Y. enterocolitica* was not decontaminated by washing with tap water or with water added with 60 ppm of chlorine, only the addition of 220 ppm of chlorine in the water is able to eliminate 1-2 logs of *Y. enterocolitica*. Our results have confirmed the ones obtained by Pezzuto *et al.* [22], performing similar experiments about washing leafy green vegetables spiked with *Listeria monocytogenes* and *Salmonella enterica*, using different domestic washing procedures, including hypochlorite at 200 ppm but not at 60 ppm.

The addition of 60 ppm of chlorine in industrial washing process, as well as for household washing treatments of vegetables, could be a useful tool to prevent the spread of the contamination when the microbial load is low [2]. The addition of high concentration of chlorine seems to reduce, of about 1-2 logs, the concentration of *Y. enterocolitica* as observed by other authors for their industrial use [8, 22]. However, as the addition of high concentration of chlorine (about 220 ppm) dramatically modifies the organoleptic quality of the lettuce, this procedure should be recommended only in domestic washing procedure in case of consumption of raw vegetables by high risk population, like immunocompromised people.

Washing has been identified as a potential pathway for dispersion of microorganisms from one site to another removing the bacterial from initially localized contaminated parts and spread them to the rest of non-contaminated leafy green vegetables [8]. Even if the ability of the industrial or domestic washing process to remove naturally present microorganisms from fresh-cut produce is limited (1-2 logs), the addition of low concentrations of chlorine are effective to maintain the quality of the washing water. Therefore, disinfectant dose used to avoid cross-contamination is lower compared to the dose needed for microbial inactivation in the fresh produce, so that sanitizing strategies have to be focus primarily on preventing cross-contamination in the washing tank to further implement produce safety [23].

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