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Assessing the efficacy of novel and conventional disinfectants on *Salmonella* cross contamination during washing of fresh-cut lettuce and their impact on product shelf life

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ABSTRACT

The effect of the application of different disinfectants on the microbial load and sensory quality of fresh-cut lettuce was evaluated during washing, and after subsequent storage at 4 °C under modified atmosphere packaging (MAP). Different families of potential alternative sanitizers were tested: quaternary ammonium compounds (QACs) as benzalkonium chloride (BZK), and didecyldimethylammonium chloride (DDAC); isothiazolinones (mixture of chloromethylisothiazolinone and methylisothiazolinone, CMIT:MIT 3:1); and, essential oils (carvacrol, CAR). All these disinfectants were effective to inactivate *Salmonella* (10^3 CFU/mL) present in wash water. In addition, all tested chemicals could reduce *Salmonella* on produce to levels >95%, with chlorine and BZK-CAR reaching maximum reductions of 99.0%. These disinfectants also enhanced a reduction in natural microbiota present on the produce. The highest reduction corresponded to free chlorine (50 mg/L) (95.1%), CMIT:MIT (50 mg/L) (Kathon®) (94.5%), and BZK (300 mg/L) (91.3%). However, only free chlorine (50 mg/L), CMIT:MIT (50 mg/L) (Kathon®), and DDAC (100 mg/L) resulted in minimal negative impact on end-product quality during 14-day storage. On the contrary, an adequate sensory quality could be only maintained up to 7 days for produce treated with BZK (300 mg/L).

1. Introduction

Consumption of fresh-cut vegetables (minimally processed) has increased in recent years as they satisfy consumer demands for healthy and easy-to-use products (Artés 2004; Wiley 1994). However, since fresh produce is eaten raw, it has become widely recognized as a vehicle for transmitting foodborne illness outbreaks (CDC, 2017; Murray et al., 2017), even to a greater extent than any other single category of food.

The immersion of fresh-cut vegetables in water for washing is a common practice in the vegetable industry, aimed to eliminate or reduce dirtiness, pesticides and microbial contamination. However, repeated cycles of washing typically decrease water quality. Thus, the potential cross-contamination of pathogens between produce batches, and between contaminated wash water and produce can take place in the washing tank. To minimize or reduce cross contamination, disinfectants are applied, being chlorine and derivatives the most commonly used sanitizers. However, the efficiency of chlorine as a sanitizer to inactivate microorganisms on fresh-cut produce is generally limited to 1- to 2-log reductions, being complete reduction commonly unachievable (Banach et al., 2015; Van Haute et al., 2013a; Ölmez & Kretzschmar, 2009). These limitations are related to microbial attachment to surfaces, crevices and cut edges together with biofilms formed by microorganisms (Sapers, 2001). Other disadvantage derived from the use of chlorine is the production of potential carcinogenic halogenated disinfection by-products (DBP's) in wash water when organic matter is present. In this respect, there is a current trend to ban the use of chlorine-based sanitizers during washing processes in Europe (Banach et al., 2015; Ölmez & Kretzschmar, 2009). As a result, there is a need to look for alternative sanitizers to be used during the washing of fresh-cut vegetables (Artés-Hernández, 2017; Banach et al., 2015; Gil & Allende, 2018; Gil et al., 2009; Murray et al., 2017; Oms-Oliu et al., 2010; Ölmez & Kretzschmar, 2009).

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According to European (Council Regulation, 2011) and U.S. (Code of Federal Regulation. Part 175, Title 21, Volume 3., 2020) regulations, isothiazolinone-derived biocides are authorized to be used as antimicrobials in food contact materials. They may be used as sanitizers in food processing industries. The most commonly used isothiazolinone biocides consist of a 3:1 mixture made of 5-chloro- and 2-methyl-4-isothiazolin-3-one (CMIT:MIT) sold commercially as Kathon® or Predator 8000®. The main inactivation mechanism of isothiazolinones is based on the inhibition of thiol-containing cytoplasmatic and membrane-bound enzymes, leading to the inhibition of bacterial metabolism (Williams 2007).

Quaternary ammonium compounds (QACs) are cationic antibacterial agents widely used as disinfectants in food processing industries (Gilbert & Moore, 2005; Yoshimat & Hiyama, 2007). Their antibacterial activity is highly dependent on the chain length of alkyl groups and on the degree of C–C saturation, affecting their hydrophobicity and interaction with bacteria (Gilbert & Moore, 2005). Positively charged molecules are contained in the long-chain alkyl group of C12–C16 in benzalkonium chloride (BZK). These positive molecules strongly interact with the anionic sites found on the bacterial cell wall, resulting in the collapse of membrane permeability, causing cell lysis. The structure of twin long-chain of alkyl groups of C22 of didecyldimethylammonium chloride (DDAC) has been reported to cause a strong antimicrobial activity (Yoshimat & Hiyama, 2007).

Some antimicrobials of increasing interest correspond to plant essential oils (EOs) due to their potential as natural food preservatives and they are Generally Recognized As Safe (GRAS) (Food and drug administration, food for human consumption, 2020). Monoterpenes as carvacrol (CAR) solutions have shown great antimicrobial effects on different fresh-cut fruit and vegetables (Artés-Hernández, 2017). Their antimicrobial activity has been linked to their phenolic hydroxyl group, which acts as a proton exchanger and destabilizes the cytoplasmic membrane, causing cell lysis (Sun, 2014).

The main objective of the present work was to evaluate the effectiveness of these alternative sanitizers such as CMIT:MIT (Kathon® and Predator 8000®), DDAC, BZK, and CAR, in comparison with free chlorine in terms of preventing cross-contamination of *Salmonella* during the washing step of lettuce, assessing the sensory changes produced by such chemicals during produce storage.

2. Experimental

2.1. Bacterial strain

Salmonella enterica serovar Thompson strain (LFMFP 687) provided by Prof. F. Pérez-Rodríguez (Universidad de Córdoba) was used. The strain had previously been transformed with a pGT-Kan mB156 plasmid containing the green fluorescent protein gene (GFP) and a gentamicin resistance gene. Stock culture was stored in cryovials on Trypticase Soy Broth (TSB, Scharlab, Spain) supplemented with 15 μg/mL of gentamicin (TSB with gentamicin) with 20% glycerol at −20 °C. Working cultures were prepared from a cryovial containing the stock culture in 20 mL of the same supplemented media at 37 °C under rotary shaking for 24 h. Fresh bacterial cultures of ca. 10^9 colony forming units (CFU)/mL at stationary phase were obtained. This concentration was confirmed by plate count enumeration. The cells were separated from the media by centrifuging at 3500 r/min (Consul 21-R, Orto-Alresa, Scharlab, Spain) for 25 min and resuspended in 5 mL of sterilized saline solution (NaCl, 0.85%) (Scharlab, Spain).

2.2. Antimicrobial solutions

Antimicrobials from different compound families were studied, corresponding to QACs as Benzalkonium chloride (BZK) (CAS 63449-41-2) and didecyldimethylammonium chloride (DDAC) (CAS 7173-51-5) (Sigma-Aldrich, Germany); isothiazolones from two different commercial brands, that is, Kathon® (Dow®, The Netherlands) and Predator 8000® (Innospec Limited, Germany) containing CMIT:MIT (5-chloro- and 2-methyl-4-isothiazolin-3-one) in 3:1 ratio at a final concentration of 1.5% total active ingredient; carvacrol (CAR) (CAS 499-75-2) (Sigma-Aldrich, Germany); and sodium hypochlorite solution (NaOCl) (containing 4.00–4.99% active chlorine) (CAS 7681-52-9) (Sigma-Aldrich, Germany). Colorimetric determination of free chlorine concentration in NaOCl solutions was tested by using a chemical test kit HI93701-01 (Hanna Instruments®, Spain).

Stock solutions of 2000 mg/L for DDAC; 1000 mg/L for CMIT/MIT (3:1) and BZK; 500 mg/L for CAR; and free chlorine were prepared in deionized water and stored in dark at 4 °C for seven days. CAR solution was prepared using sterilized water containing 1.0% (v/v) of polysorbate 80 (Panreac, Spain) to allow mixing. All solutions were prepared prior to use.

All concentrations studied were chosen as minimum bactericidal concentration (MBC) values, determined in a previous work simulating real conditions of the fresh-cut industry using simulated wash water (see Section 2.3) contaminated with *Salmonella* at 10^3 CFU/mL, and applying a contact time of 90 s (Pablos et al., 2018). According to this study, MBC values corresponded to 100, 30, 50, 300, and 9 mg/L for BZK, DDAC, CMIT:MIT (3:1), CAR, and free chlorine, respectively.

2.3. Simulated vegetable wash water preparation

Simulated wash water (SWW) was prepared based on a previous physico-chemical characterization of different wash water in a Spanish vegetable processing industry (Pablos et al., 2018). The baseline SWW was generated to reproduce the following parameters: TOC (Total Organic Carbon) = 150 mg/L, turbidity = 100 NTU (Nephelometric Turbidity Unit), conductivity = 1000 μS/cm, and pH = 6.2. The TOC value was obtained by adding malt extract for microbiology (Appli. Chem. Panreac) (350 mg/L); and turbidity was adjusted by supplementing kaolin powder (Merck) (100 mg/L) dissolved 24 h prior to use. Total organic carbon (TOC) was estimated using a combustion/non-dispersive infrared gas analyser model Shimadzu TOC-V (Mettler Toledo, Spain). pH and conductivity were measured by a benchtop Crison GLP 22 pHmeter and Crison EC-Meter-Basic 30+ conductimeter, respectively (Scharlab, Spain). Turbidity was measured by nephelometry following the standardized APHA methods (Standard Methods 2130 B) (Rice et al., 2012) using a Hanna Instruments HI 88703 turbidimeter (Scharlab, Spain).

Additional experiments were carried out increasing TOC values up to 500 mg/L in SWW by increasing malt extract concentration up to 1475 mg/L. The adsorption capacity of organic matter in water may play a significant role in bacterial attachment, which may reduce the efficacy of the disinfectant against bacterial inactivation. Obtained turbidity and conductivity values agreed with average values reported by López-Gálvez et al. (2019). The SWW chemical profile corresponded to $F^- = 0.14$ mg/L, $Cl^- = 282$ mg/L, $NO_2^- = 0.030$ mg/L = , $Br^- = 10.2$ mg/L, $NO_3^- = 51.6$ mg/L, $SO_4^{2-} = 51.0$ mg/L, $Na^+ = 87.7$ mg/L, $NH_4^+ = 1.24$ mg/L, $K^+ = 108.0$ mg/L, $Mg^{2+} = 9.55$ mg/L, $Ca^{2+} = 47.1$ mg/L.

2.4. Simulation of fresh-cut lettuce washing with different sanitizers

Iceberg lettuce (*Lactuca sativa* L.) was purchased from a local supermarket (Madrid, Spain) and stored at 4 °C for 4 day as maximum. After discarding the outer leaves, remaining leaves were pulled out, and carefully cut into pieces of ca. 12 cm^2 with a knife previously disinfected with ethanol and left to air dry until use. The washing process was performed in a flask with 1 L SWW, cooled at 4–7 °C.

Before washing, SWW was inoculated with *Salmonella* to obtain a concentration of 10^3 CFU/mL. Plate count method was performed for enumeration. Then, 25g-portions of uninoculated fresh-cut lettuce were introduced in the flask resulting in a produce:water ratio of 1:40 (w:v). No *Salmonella* was initially present in the produce samples. To simulate the washing process, lettuce pieces were stirred by using magnetic-stirring at 260 rpm for 90 s.

Another type of experiments was performed following the same procedure although in this case, once the lettuce samples were washed with SWW inoculated with *Salmonella*, they were washed again, after adding the appropriate disinfectant solution to the 1 L flask. The disinfection process was quenched by adding 1 μL/mL sodium thiosulfate (0.05 M) (CAS 7772-98-7, Fluka, Germany). It was confirmed that the pH of SWW suspensions after the addition of each antimicrobial was not affected, ranging between 6.25 ± 0.75.

After the washing step in both type of experiments, processed 25 g-samples of fresh-cut lettuce were de-watered by spin-drying in a hand-held salad spinner for 1 min and, subsequently, cool air was applied for another minute. Next, samples were placed in stomacher bags containing 225 ml buffered peptone water (BPW) and homogenized for 2 min at 8 strokes/s in Stomacher Mixer 400 (Scharlab, Spain). The homogenized liquid samples were later used to quantify microbial contamination in lettuce pieces. Bacterial quantification was performed as explained in Section 2.6. The experiments were performed threefold, in different days, in order to capture process and biological variability.

2.5. Calculations of bacterial reduction and water-to-lettuce transfer ratio (WLTR)

The effect of washing with antimicrobial on the populations of the inoculated *Salmonella* and aerobic mesophilic bacteria naturally present in lettuce was evaluated for each experiment in wash water and produce, estimating the difference in % and log scale of microbial counts, before and after washing, and with and without disinfectant.

Besides this, cross-contamination in fresh-cut lettuce was evaluated by quantifying *Salmonella* transfer from inoculated water to lettuce in the second washing cycle. A water-to-lettuce transfer ratio (WLTR) was calculated based on Eq. (1) provided by Holvoet et al. (2014) for other microorganisms. This equation represents the fraction between the *Salmonella* counts quantified on lettuce after washing and the potential maximum *Salmonella* level, which is defined as the total number of inoculated *Salmonella* cells in the water, expressed in gram of lettuce.

$$\mathrm{WLTR} = \frac{\mathrm{Log}\ (\textit{Salmonella}\ \text{load in lettuce after washing})\ (\mathrm{CFU\ g^{-1}})}{\mathrm{Log}\ \left(\frac{\textit{Salmonella}\ \text{in water before washing}\ \left(\mathrm{CFU\ mL^{-1}}\right) \times \text{Volume of water (mL)}}{\text{Weight of lettuce (g)}}\right)(\mathrm{CFU\ g^{-1}})} \tag{1}$$

2.6. Microbiological analyses

Serial dilutions of 100 μL of the homogenized liquid samples were performed in 900 μL of sterile saline solution (0.85%) (Scharlab, Spain) in Eppendorf tubes. 10 μL of each decimal dilution were plated onto Trypticase Soy Agar (TSA) for aerobic mesophilic bacteria (APC, aerobic plate counts) quantification, and TSA containing 15 μg/mL gentamicin for *Salmonella* quantification. Also, 100 μL and 1000 μL of undiluted liquid samples were plated leading to a Limit of Quantification (LOQ) of 10^0 CFU/mL in SWW, and to a Limit of Quantification (LOQ) of 10^1 CFU/g in produce. In all cases, samples spotted onto agar plates were incubated for 24 h at 37 °C. Bacterial enumeration was performed in triplicate. For *Salmonella* enumeration, colonies with Green-fluorescence were identified under UV light (365 nm) and enumerated. APC were identified as colonies not presenting Green-fluorescence under UV light.

2.7. Packaging and storage conditions

Processed (washed) lettuce samples (25 g) were aseptically packaged into individual plastic bags (PA/PE/PA/PE, Innopack, Spain) using a chamber vacuum packaging machine (EVT-350/20, Irimar, Spain) and generating a modified atmosphere with the following gas composition: 90% N_2, 5% CO_2, 5% O_2. Changes in O_2 and CO_2 concentrations within packages were not significant after 14 days of storage. The plastic bag dimensions were 200 × 300 mm and 80 μm thickness. The O_2 permeability was 50 $cm^3/(m^2/d)$ at 23 °C and 75% Relative Humidity (RH), and the CO_2 permeability corresponded to 150–250 $cm^3/(m^2/d)$ at 23 °C and 100% RH. Packaged samples were stored at 5 °C for 14 days and extracted for analysis at day 0 (the day experiments were performed), 5, 7, and 14. In each sampling point, samples from each treatment were analyzed for sensory properties and enumeration of APC and *Salmonella*. The bacterial increase of *Salmonella* and APC during storage was estimated as the difference, in log scale, between the concentration at day 14 (final) and 0 (initial concentration).

2.8. Sensory evaluation of visual and organoleptic quality

A panel of four trained personnel examined packaged fresh-cut lettuce after processing (day 0) and during product storage. Samples were coded with random numbers to minimize subjectivity and to ensure test accuracy. Sensory quality was evaluated in relation to the possible effect of disinfectants on the visual and odour quality of produce.

The visual analysis concerned specifically the degree of browning (i.e., areas with brown and yellowish colour) which was evaluated under white light using a five-point scale from 1 (most browned) to 5 (least brown). Descriptions for each score were as follows: 1 = extremely poor, 2 = poor, 3 = fair (limit of consumer acceptability), 4 = good, 5 = excellent.

The intensity of the disinfectant off-odour was evaluated immediately after opening the packed samples, on the same scale range with 1 and 5 representing for highest and lowest intensity for the used disinfectant, respectively; where 1 = severe, 2 = strong, 3 = moderate (limit of consumer acceptability), 4 = slight, and 5 = none. Standard deviation (SD) error of the same sample among panellists corresponded to 0.3.

For statistical purposes, the slope from the curves of mean sensory scores over time was calculated and used to represent the deterioration rate of visual and odour quality of the produce. Colorimetric measurements were also performed but results were not conclusive. Detail of experimental procedure and results are presented in Supplementary Material, Section S2 (S2.1) and Tables S1–S6.

2.9. Statistical analysis

Microbiological counts were transformed into decimal logarithmic scale when needed and processed using procedures and equation described in Section 2.5. The limit of quantification for *Salmonella* and APC corresponded to 10^0 CFU/mL and 10^1 CFU/g. The treatment of the microbiological data and data processing and estimation of the slope for the linear trend of sensory analysis data were performed with Microsoft Excel (Microsoft, Redmond, WA, USA). In addition, ANOVA and MANOVA analyses were performed on microbiological and sensory data. A confidence level of 95.0% (α = 0.05) was applied. Thus, data were

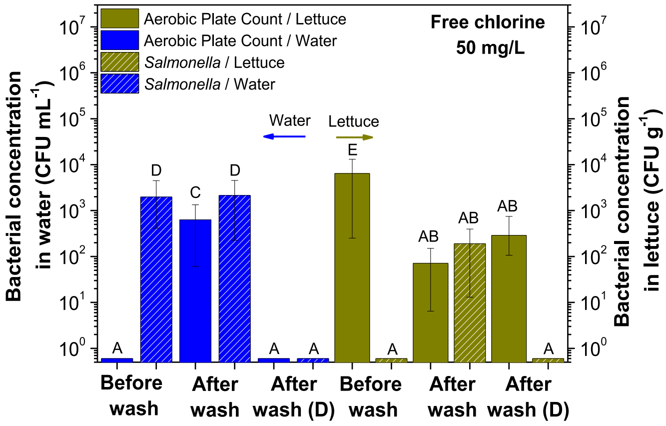

Fig. 1. Concentrations of *Salmonella* and Aerobic Plate Count (APC) estimated in produce and water before and after treatment of fresh-cut lettuce (260 rpm, 90 s, 4 °C) with simulated wash water (SWW, TOC 150 mg/L) inoculated with *Salmonella* (10^3 CFU/mL) without disinfectant and disinfectant (D) with free chlorine at 50 mg/L. Limit of Quantification (LOQ) in wash water: 10° CFU/mL. Limit of Quantification (LOQ) in produce: 10^1 CFU/g. Significant differences ($p \leq 0.05$) between treatments from Tukey's HSD test have been indicated with a different uppercase capital letter, with "A" assigned to the lowest value.

considered significantly different at $p \leq 0.05$. The statistical tests considered as factors "type of disinfectant", "disinfectant level" and "TOC level" and as dependent variables, "*Salmonella* reductions in water" and "*Salmonella* transfer to lettuce during washing (%)", "APC reductions in lettuce during washing (%)", "APC increase during storage (log)" and "the slope corresponding to deterioration rate showed by sensory data". Significant differences ($p \leq 0.05$) among microbial data and sensory quality between treatments from Tukey's HSD test have been indicated with a different uppercase capital letter, with "A" assigned to the lowest value in Figures and Tables. The statistical treatment was performed by means of SPSS 8.0 software (SPSS Inc. Chicago, Il, USA).

3. Results & discussion

Overall, results showed that the presence of disinfectant could reduce microbial counts significantly, both in water and lettuce during washing ($p \leq 0.05$). However, disinfectant type and organic matter level did not significantly affect *Salmonella* and APC reductions in water and lettuce according to the statistical analysis ($p > 0.05$). By contrast, as for sensory quality, deterioration rate was statistically affected by both disinfectant and disinfectant level ($p \leq 0.05$), with non-disinfectant and chlorine showing the lowest rate. The impact of disinfectant level was especially evident for CMIT:MIT (Predator 8000®) which is the one with higher number of levels tested. In below sections, main outcomes from each disinfectant tested are presented, showing relevant results for microbial and sensory analyses.

3.1. Free chlorine

Cross contamination levels by *Salmonella* in water and lettuce without and with chlorine are shown in Fig. 1. For the other disinfectants tested, these results are shown in Figs. S1–S5, Supplementary Material. When the non-inoculated lettuce was immersed and washed for 90 s in 1 L of contaminated wash water without disinfectant (ca. 10^3 CFU/mL), *Salmonella* concentration on lettuce could reach levels as high as ca. 10^2 CFU/g. Also, a high transfer of APC could be observed from the produce to the wash water. However, the addition of chlorine during washing was able to lower counts of *Salmonella,* in both wash water and the produce, below the quantification limit. APC were not quantified in wash water, while it were able to remain on produce which is in agreement with findings of Banach et al., 2020.

The effect of two levels of organic matter and free chlorine in SWW on the reduction of *Salmonella* in water and produce are shown in Table 1. The total reduction of *Salmonella* in SWW was achieved at both disinfectant concentration levels (10 and 50 mg/L). At the highest organic matter load (TOC = 500 mg/L), total inactivation of *Salmonella* was obtained by using 50 mg/L of free chlorine. As expected, without adding disinfectant, *Salmonella* remains in the wash water. Moreover, the addition of free chlorine was able to prevent *Salmonella* transfer to the produce. In addition to this, the presence of the disinfectant in wash water led to reductions of natural aerobic bacteria on the produce higher than 90% (1 log). However, as reported by Banach et al., 2015; Ölmez & Kretzschmar, 2009; Van Haute et al., 2013a; and Sapers 2001, it is likely that the differences in the natural aerobic bacterial initial concentration

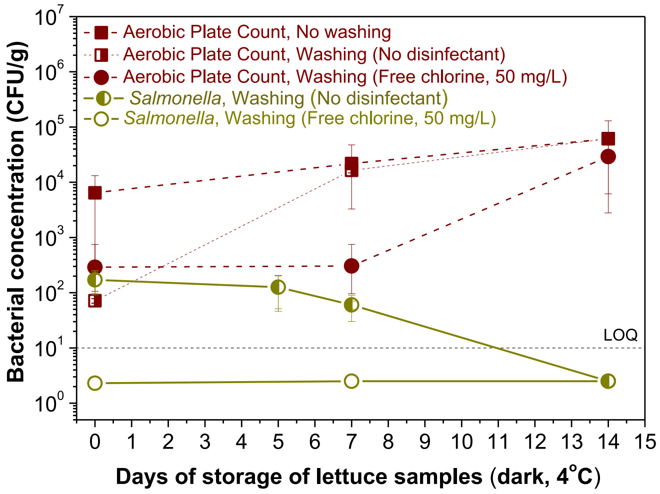

Fig. 2. Concentration of *Salmonella* and Aerobic Plate Count naturally present (APC) in produce, before and after washing fresh-cut lettuce in simulated wash water. 1 L of SWW previously inoculated with *Salmonella* (10^3 CFU/mL). TOC: 150 mg/L. Washing: 260 rpm, 90 s, 4 °C. Disinfectant: Free chlorine 50 mg/L.

Table 1
Reduction of *Salmonella* and Aerobic Plate Count naturally present in produce (APC) during washing. Effect of free chlorine concentration (10 and 50 mg/L) and organic matter content in wash water.

Bacterial reduction ± SD (%)[a]	TOC[c] 150 mg/L (SWW)		TOC[c] 150 mg/L (SWW)		TOC[c] 500 mg/L (SWW)	
	No disinfectant	Free chlorine (10 mg/L)	No disinfectant	Free chlorine (50 mg/L)	No disinfectant	Free chlorine (50 mg/L)
APC on lettuce	0.00 ± 11.1	97.1 ± 1.6	81.2 ± 18.8	95.1 ± 0.6	8.00 ± 11.1	78.0 ± 11.1
Salmonella [b] on lettuce	–	>99.0	–	>99.0	–	>99.0
Inoculated *Salmonella* in SWW	16.3 ± 12.5	>99.9	8.30 ± 11.7	>99.9	6.20 ± 12.5	>99.9

–No *Salmonella* initially present in produce.

[a] Values are the mean of at least three replicates ± standard deviation.

[b] *Salmonella* inoculated (10^3 CFU/mL) in 1 L simulated wash water (SWW). Washing at 260 rpm, 4 °C, 90 s contact time.

[c] Total organic carbon.

Table 2
Sensory evaluation of the differences between the smell and appearance of the produce treated with free chlorine and in absence of disinfectant on the day of treatment (day 0), 7th and 14th day of storage in darkness at 4 °C in MAP after washing in SWW, 260 rpm, 90 s contact time, 4 °C.

Sensory quality	No disinfectant, TOC 150 mg/L			Free chlorine (50 mg/L), TOC 150 mg/L		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Colour	5.0	4.8	5.0	5.0	4.8	4.7
Odour	5.0	4.8	4.8	4.8	4.8	4.7

Table 3
Sensory evaluation of the differences between the smell and appearance of the produce treated with CMIT:MIT (3:1) (Kathon®) and in absence of disinfectant on the day of treatment (day 0), 7th and 14th day of storage in darkness at 4 °C in MAP after washing in SWW, 260 rpm, 90 s contact time, 4 °C.

Sensory quality	No Disinfectant, TOC 150 mg/L			CMIT:MIT (50 mg/L), TOC 150 mg/L		
	Day 0	Day 7	Day 14	Day 0	Day 7	Day 14
Colour	5.0	4.7	4.7	5.0	4.2	4.3
Odour	5.0	4.0	4.7	5.0	3.7	4.3

on the produce and its distribution on crevices, creases, pockets, and natural openings in the vegetable tissues contributes to the overall lack of effectiveness of chlorine in inactivating bacteria on the produce. In fact, according to statistical analysis, reduction of APC on lettuce after washing without and with disinfectant (D) revealed non-significant differences. Thus, these data are not presented for the following disinfectants (but shown in Supplementary Material, Table S7).

Salmonella and APC quantified on the produce before and after washing at day 0, 7 and 14 during storage are shown in Fig. 2. In lettuce samples washed with chlorinated water, *Salmonella* remained uncountable in the final product throughout storage. *Salmonella* neither grew nor recovered. On the contrary, APC was able to increase up to 4–5 log CFU/g as APC remained in the produce after the washing in presence of the disinfectant. The growth was faster for the produce washed without disinfectant. Moreover, non-washed samples exhibited a more limited growth potential as the initial concentration was closer to the maximum population density. Statistical tests confirmed that there were non-significant differences ($p > 0.05$) for the total APC increase during storage (log) at the organic matter and chlorine levels assessed. Several authors (Gil et al., 2009) have also confirmed that microbial populations of fresh-cut vegetables seem to increase rapidly and even are able to exceed the initial level on the wash water during extended storage.

The effect of washing the produce in presence of free chlorine in terms of sensory qualities is depicted in Table 2. Based on sensory analysis, no differences on general appearance and odour were found out in the produce washed in presence of free chlorine in comparison with the treatment without adding disinfectant after both, 7 and 14 days of storage ($p > 0.05$). Thus, the growth of APC estimated in the produce during storage does not contribute to deteriorate the overall sensory quality of any sample which is in agreement with findings of Van Haute et al., 2013b.

Similarly, the increase in the TOC of SWW up to 500 mg/L did not lead to significant differences in the colour and odour of the produce due to the addition of free chlorine (50 mg/L) during washing, ($p > 0.05$) (Table S10, Supplementary Material). Thus, after 7 and 14 days of storage, the sensory quality of the produce was not affected, reaching a score of 3.8/4.7 (colour/odour, 7 days), 3.7/4.7 (colour/odour, 14 days) when using free chlorine as disinfectant, and 3.5/4.7 (colour/odour, 7 days), 4.7/4.8 (colour/odour, 14 days) when no disinfectant was added.

3.2. Isothiazolinones

The addition of CMIT:MIT yielded 99.9% reduction of *Salmonella* concentration in SWW at both levels of organic matter in water (Table S8, Supplementary Material). It is remarkable that all disinfectants tested led to 99.9% (3-log) reduction of *Salmonella* concentration in SWW at both levels of organic matter in water. A total inactivation in wash water has been considered as better intervention step to prevent product contamination (Luo et al., 2011; Murray et al., 2017; Tomás-Calleja et al., 2012; Van Haute et al., 2013a). As reported by Ölmez & Kretzschmar (2009), this fact is important not only for microbial safety reasons, but also for economic and environmental factors, as reusing of water may become a common practice for the industry to avoid the high amount of water discharged.

Despite both CMIT:MIT brands (i.e. Kathon® and Predator 8000®) produced similar *Salmonella* inactivation levels in wash water, CMIT:MIT (3:1) (Predator 8000®) did not seem to be fully efficient against *Salmonella* on the produce. An increase of the disinfectant concentration up to 300 mg/L of CMIT:MIT (3:1) (Predator 8000®) was required to reach a reduction of *Salmonella* on lettuce of 96.7 ± 3.0% (ca. 2 log) at low organic load. Lower disinfectant levels of CMIT:MIT (3:1) (Predator 8000®) (50, 100 and 200 mg/L) resulted in lower reductions on lettuce, corresponding to 0.80 ± 0.4; 10.7 ± 3.0; and 41.4 ± 3.0% respectively (Table S9, Supplementary Material). On the contrary, disinfectant levels of CMIT:MIT (3:1) (Kathon®) of 50 mg/L dropped the pathogen concentration on lettuce up to 82.1 ± 14.8% (less than 1-log reduction) (Table S9, Supplementary Material). These results showed that even in presence of the antimicrobial, *Salmonella* in wash water was able to contaminate and persist on lettuce after washing, which highlights again the necessity of performing intervention steps in wash water to prevent product contamination.

Increasing the organic matter in wash water (500 mg/L TOC) seemed to diminish the efficiency of CMIT:MIT as sanitizer, leading to a lower reduction of *Salmonella* concentration on the produce, which corresponded to 34 ± 12.5% (Table S9, Supplementary Material).

The results of the sensory analysis (colour and odour) of the produce washed in presence of CMIT:MIT (3:1) are included in Table 3. Score values given by panellists showed that sensory quality of the produce washed in presence of CMIT:MIT (3:1) (Kathon®) (50 mg/L) was still accepted by consumers after 14 days of storage.

Similar results were found out when the organic matter content was higher (TOC = 500 mg/L). In this case, the overall sensory quality of the produce was again within acceptable values after 7 and 14 days of storage; reaching a score of 4.7/5.0 (colour/odour, 7 days), 3.3/4.7 (colour/odour, 14 days) when using CMIT:MIT (3:1) (50 mg/L) (Kathon®) as disinfectant, and 4.8/5.0 (colour/odour, 7 days), 4.7/4.7 (colour/odour, 14 days) when no disinfectant was added (Table S10, Supplementary Material).

In contrast, CMIT:MIT (3:1) (300 mg/L, Predator 8000®) did lead to sensory rejection of the produce after 14 days as scores for colour and odour were below the acceptability threshold (i.e., 2.2 and 2.5, respectively). Although after 7 days of storage, produce is still within the acceptability range (i.e., 3.2/3.4 for colour/odour), its score was much lower compared to that obtained for the washed produce without disinfectant (5.0/4.8, colour/odour) (Table S10, Supplementary Material). Thus, although a higher amount of sanitizer in wash water may be required to effectively inactivate *Salmonella* on the produce, it affects the sensory quality of the produce. This result was also reported by findings of López-Gálvez et al., 2009.

APC on treated produce (washed and washed in presence of CMIT:MIT (3:1) (Kathon®) (50 mg/L) was able to grow during storage exceeding initial values present in the produce before washing (Fig. S6, Supplementary Material). This bacterial growth did not lead to any change in appearance of the produce throughout storage for 14 days. Similar results were obtained when increasing the organic load of the SWW up to TOC values of 500 mg/L (data not shown). Thus, although differences in bacterial inactivation during washing were observed due to differences in quality of the wash water, no differences after storage

Table 4
Reduction of *Salmonella* on produce (previously transferred from wash water during washing in absence of disinfectant) during washing in presence of disinfectant. Effect of concentration of QAC based disinfectants and organic matter content in wash water on bacterial inactivation.

Bacterial reduction ± SD [a] (%)	TOC[c] 150 mg/L (SWW)						TOC 500 mg/L (SWW)	
	No disinfectant	BZK (300 mg/L)	No disinfectant	DDAC (50 mg/L)	No disinfectant	DDAC (100 mg/L)	No disinfectant	DDAC (100 mg/L)
Salmonella [b] on lettuce	–	66.7 ± 12.5	–	84.6 ± 12.5	–	98.3 ± 1.6	–	79.0 ± 12.5

–No *Salmonella* initially present in produce.
[a] Values are the mean of at least three replicates ± standard deviation.
[b] *Salmonella* inoculated (10^3 CFU/mL) in 1 L simulated wash water (SWW). Washing at 260 rpm, 4 °C, 90 s contact time.
[c] Total organic carbon.

Table 5
Sensory evaluation of the differences between the smell and appearance of the produce treated with BZK and DDAC and without disinfectant on the day of treatment (day 0), 7th and 14th day of storage in darkness at 4 °C in MAP after washing in SWW, 260 rpm, 90 s contact time, 4 °C, TOC: 150 mg/L.

Sensory quality	No Disinfectant		BZK (300 mg/L)		No Disinfectant		DDAC (100 mg/L)	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
Colour	4.7	3.3	3.7	2.7	4.5	4.5	3.3	3.2
Odour	5.0	3.3	3.7	1.8	5.0	4.7	4.0	3.4

Day 0: 5/5 (colour/odour).

Table 6
Reduction of *Salmonella* on produce (previously transferred from wash water during washing in absence of disinfectant) during washing in presence of disinfectant. Effect of CAR and its combination with BZK on bacterial inactivation.

Bacterial reduction ± SD [a] (%)	TOC 150 mg/L (SWW)			
	No disinfectant	CAR (300 mg/L)	No disinfectant	BZK-CAR (75–200 mg/L)
Salmonella[b] on lettuce	–	98.3 ± 1.6	–	>99.0

–No *Salmonella* initially present in produce.
[a] Values are the mean of at least three replicates ± standard deviation.
[b] *Salmonella* inoculated (10^3 CFU/mL) in 1 L simulated wash water (SWW). Washing at 260 rpm, 4 °C, 90 s contact time.

were determined in terms of microbial counts and appearance, which is in agreement with the findings of Allende et al., 2008.

3.3. Quaternary ammonium compounds (QACs)

Salmonella in wash water seemed to be highly susceptible to this type of compounds since effective inactivation was achieved in water for all studied concentration levels (Table S8 in Supplementary Material). Table 4 shows the effect of QACs on the reduction of *Salmonella* on lettuce leaves. The highest *Salmonella* reduction was obtained with DDAC at levels of concentration of 100 mg/L. As expected, DDAC demonstrated a stronger antimicrobial effect than BZK. Lower concentration values of DDAC (50 and 100 mg/L) led to higher reductions in bacterial counts than those obtained by using BZK at higher values of concentration (300 mg/L). An increase in organic matter led to a reduction of the disinfectant efficiency as shown in Table 4, as it was previously reported (Banach et al., 2015). It is important to consider the high reactivity of the disinfectant with the organic matter present in water, leading to lower efficiency in the disinfection process. Kinetics is highly dependent on disinfectant dose, contact time, and physicochemical properties of the wash water. As contact time is extremely short during produce washing, disinfectant dose has to be able to inactivate pathogens immediately. Moreover, disinfectant dose must be adjusted since organic matter built-up throughout the washing process in the tank, reducing the effectiveness of the disinfectant. Therefore, disinfectant dose is critical to a particular process to guarantee a residual disinfectant effect, preventing cross-contamination (Gombas et al., 2017).

Table 7
Sensory evaluation of the differences between the smell and appearance of the produce treated with CAR and CAR-BZK and without disinfectant on the day of treatment (day 0), 5th and 7th day of storage in darkness at 4 °C in MAP after washing in SWW, 260 rpm, 90 s contact time, 4 °C, TOC: 150 mg/L.

Sensory quality	No Disinfectant		CAR (300 mg/L)		No Disinfectant		BZK-CAR (75–200 mg/L)	
	Day5	Day 7	Day 5	Day 7	Day 5	Day 7	Day 5	Day 7
Colour	4.7	4.2	3.7	2.5	4.0	3.7	1.0	1.0
Odour	5.0	4.2	4.2	4.2	4.0	4.0	2.0	2.0

Day 0: 5/5 (colour/odour).

QACs influenced sensory quality of the end-product, but only BZK (300 mg/L) led to produce rejection after 14 days of storage mainly due to an unpleasant odour detected (Table 5). Although the score regarding the colour of the produce treated with BZK decreased, the differences with the control were not large compared with odour quality decrease. In contrast, the use of DDAC at the highest level (100 mg/L) resulted in sensory quality levels of stored products within the acceptability range. The increase of the organic content up to 500 mg/L in wash water with DDAC (100 mg/L) exhibited limited impact on the visual appearance of the washed produce, with similar deterioration rates to those observed in TOC 150 mg/L (Table S10, Supplementary Material).

Microbial counts during storage shown similar trends explained in Fig. 2 and Fig. S6 for both disinfectants (Figs. S7–S8, Supplementary Material).

3.4. Monoterpenes

Bacterial inactivation levels observed in wash water when using CAR as disinfectant are presented in Table S8 in Supplementary Material. The addition of CAR in wash water led an effective elimination of *Salmonella* in wash water. whereas inactivation levels of *Salmonella* on the produce achieved ca. 2-log reduction, as it is shown from values in Table 6. CAR seemed to be one of the most effective disinfectants evaluated as alternative to chlorine in terms of bacterial inactivation, together with DDAC (100 mg/L). However, the sensory evaluation results, presented in Table 7, indicated that the disinfectant had, in general, a great impact on the visual appearance of the produce within 7 days of storage causing leaf spots. No off-odours were detected.

Regarding the addition of CAR in combination with BZK at lower levels of concentration in wash water led to an effective inactivation of *Salmonella* in both, wash water (Table S8) and on produce (Table 6), showing similar efficiency to that of free chlorine, in terms of bacterial inactivation. Nonetheless, sensory evaluation of the produce (Table 7) led to the rejection of the produce after 7 days of storage.

Similar trend in microbial counts evolution throughout storage was

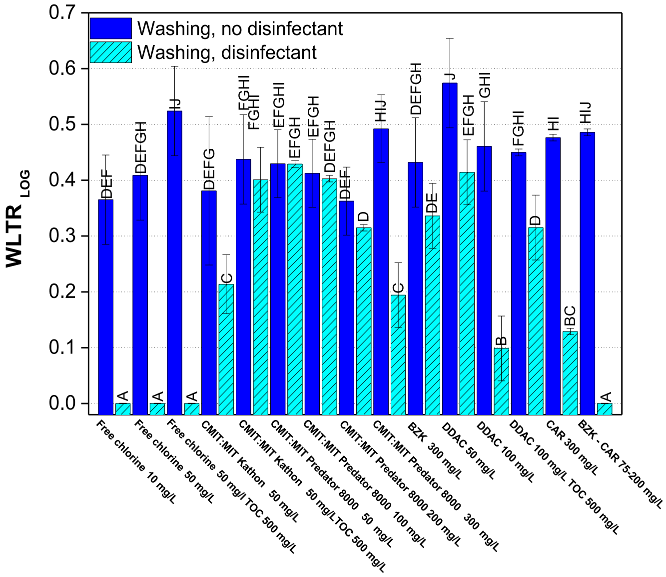

Fig. 3. Water-to-Lettuce Transfer Ratio (WLTR) during washing fresh-cut lettuce in simulated wash water. Effect of evaluated disinfectants. 1 L of SWW previously inoculated with *Salmonella* (10^3 CFU/mL). TOC: 150 mg/L (if not otherwise indicated). Washing: 260 rpm, 90 s, 4 °C. Significant differences ($p \leq 0.05$) between treatments from Tukey's HSD test have been indicated with a different uppercase capital letter, with "A" assigned to the lowest value.

observed for this disinfectant (Figs. S9–S10, Supplementary Material) in comparison with the previous disinfectants studied. Hence, the type and dose of disinfectant did not seem to have influence in APC viability on the produce during washing and therefore, throughout storage. However, the addition of the disinfectants to the wash water may prevent pathogens as *Salmonella* from being transferred from the wash water to the produce, and therefore, from growing throughout storage.

3.5. *Water-to-lettuce transfer ratio (WLTR)*

The water-to-lettuce transfer ratio (WLTR) was calculated to evaluate cross-contamination of *Salmonella* from the artificially contaminated wash water to the fresh-cut lettuce following a similar procedure to that reported by Holvoet et al. (2014) and shown in Fig. 3. This calculation enabled us to quantify the effect of the disinfectant on preventing cross-contamination from *Salmonella* present in wash water to an uncontaminated produce. All washings of the produce in contaminated wash water led to the cross-contamination of the produce when no sanitizer was used. Conventional free chlorine sucessfully prevented the produce from cross-contamination episodes. Considering the alternative disinfectants explored in this work, the combination BZK-CAR (75–200 mg/L) was the only one that reached similar results to chlorine in terms of bacterial transfer prevention, which is in agreement with bacterial reduction shown in Tables 1 and 6, for free chlorine and BZK-CAR respectively. DDAC (100 mg/L), CAR (300 mg/L), BZK (300 mg/L), and both CMIT:MIT (3:1) Kathon® (50 mg/L) and Predator 8000® (300 mg/L) showed lower efficacy in bacterial reduction and cross-contamination prevention.

4. Conclusions

The outcomes of this research provide further evidence that the washing of the produce without sanitizers favours *Salmonella* and aerobic bacteria cross-contamination between wash water and fresh-cut produce. Thus, the potential cross contamination from *Salmonella* in wash water to the produce was successfully quantified. It has been confirmed that the application of chemical sanitizers during washing can lead to the inactivation of pathogens in wash water, but its dosage is not able to totally eliminate natural microbiota present on the produce. Hence, the effective use of chemical sanitizers during the washing step is a critical point for keeping quality and safety of produce.

Although the use of chlorine, minimizing the effective dose, still represents the most suitable chemical for microbial disinfection, this study has proven that QACs as DDAC (100 mg/L) and BZK (300 mg/L), and isothiazolinones as CMIT:MIT (3:1) Kathon® (50 mg/L) can be used as an effective alternative for the disinfection of washing water. These compounds were able to reduce bacteria in wash water to undetectable levels, thus, decreasing the potential of cross-contamination. This could be quantified with a remarkable reduction of *Salmonella* water-to-produce transfer ratio and 1-log reduction of natural microbiota on produce. Moreover, DDAC and CMIT:MIT (3:1) Kathon® allowed extending the shelf-life of the produce up to 14 days without compromising sensory quality.

CRediT authorship contribution statement

Cristina Pablos: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Aitor Romero:** Investigation, Methodology, Writing – review & editing. **Ana de Diego:** Investigation, Methodology, Writing – review & editing. **Carla Corrales:** Investigation, Methodology, Writing – review & editing. **Rafael van Grieken:** Corrales, Supervision, Writing – review & editing. **Isabel Bascón:** Investigation, Methodology, Writing – review & editing. **Fernando Pérez-Rodríguez:** Formal analysis, Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Javier Marugán:** Formal analysis, Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113441.

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