

Influence of aqueous ozone, blanching and combined treatments on microbial load of red bell peppers, strawberries and watercress

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A B S T R A C T

The effectiveness of ozone in aqueous solution treatment on microbial inactivation was studied for three combinations microorganism/food: *Listeria innocua*/red bell peppers (artificially inoculated), total mesophiles/strawberries, and total coliforms/watercress, with two concentrations (0.3 and 2.0 ppm). Blanching treatments (50–60 °C) were also individually applied and in combination with ozone, for studying possible synergistic effects. In relation to ozone treatments, the highest microbial reductions were obtained for the highest concentration with the highest treatment time (3 min). Under those conditions, *L. innocua*/peppers, total mesophiles/strawberries and total coliforms/watercress were reduced respectively 2.8 ± 0.5 , 2.3 ± 0.4 and 1.7 ± 0.4 log-cycles. However, a substantial portion of the microbial populations were reduced by water washing alone, and the presence of ozone generally added an additional reduction of 0.5–1.0 log-cycles.

If ozone at the highest concentration is used, the treatment impacts on *L. innocua*/peppers and total mesophiles/strawberries load reductions were equivalent to a blanching at 50 °C (for the same treatment times).

Combining blanching and ozone did not generate synergistic effects, and in some situations microbial reductions were lower than the ones observed when treatments were applied independently.

Keywords:

Microbial reduction
Fresh fruit/vegetable
Aqueous ozone
Blanching
Combined treatments

1. Introduction

Fruits and vegetables often contain a great diversity of microbial flora and are frequently involved in food-borne outbreaks. Since fruits and vegetables are mainly consumed uncooked or minimally-processed (such as in ready-to-eat salads), microbiological safety becomes a very important issue to minimise consumers' risks (Sagoo et al., 2003). Mesophilic microorganisms, coliforms, yeasts and moulds are microbial populations usually found in raw and minimally processed products, at concentrations ranging from 10^3 to 10^9 cfu/g, and are responsible for both quality deterioration and safety risk (Abadias et al., 2008; Tournas, 2005; Tournas and Katsoudas, 2005).

Thermal treatments are conventionally used to attain safety standards. However, undesirable sensorial and nutritional changes, such as colour degradation, softening of tissues, vitamin losses and development of unpleasant cooked flavours, may occur. These alterations, added to the increasing consumer demanding for high-quality food standards, have focused the search on new and gentle processing technologies that prolong shelf-life without the

detrimental effects caused by severe heating. Several non-thermal technologies (such as UV-C irradiation, electric pulses and high pressure) have emerged as attractive alternatives to conventional thermal processes. Applying a non-thermal technology, the negative effects caused by heat can be minimised while safe and less-perishable products can be attained. The use of ozone as a sanitizer is one of the emerging and challenging technologies with potential application in the food industry. Ozone is one of the most potent disinfectant agents, due to its powerful oxidising effect (Güzel-Seydim et al., 2004a,b; Khadre et al., 2001; Suslow, 1998). It has been recognised that ozone is suitable for many applications related to food industry. These include food surface hygiene and preservation, equipment and food plant sanitation and reuse of waste waters (Graham, 2000; Kim et al., 1999; Palou et al., 2002; Zhang et al., 2005). In 1997, US Food and Drug Administration classified ozone as Generally Recognised as Safe (GRAS) substance for use as a disinfectant or sanitizer in foods and food processing, as long as good manufacturing practices are provided (Güzel-Seydim et al., 2004b). Ozone has been found to be effective against a wide spectrum of microorganisms, including viruses, gram-negative and gram-positive bacteria, spores and fungi (Khadre et al., 2001; Manousaridis et al., 2005; Restaino et al., 1995). The required ozone concentration and the disinfection rates depend on the type of microorganism, extent of microbiological contamination,

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temperature, pH, turbidity and presence of ozone-oxidisable substances (Vaughn et al., 1987; Yuk et al., 2007). Besides its antimicrobial power, ozone gathers many other advantages which turn it into an appealing and environmental friendly technology. Ozone is a very unstable gas and its degradation product is oxygen, thus leaving no undesirable residue on food or food-contact surfaces, nor creating undesirable disinfection by-products (Mendez et al., 2003; Smilanick, 2003; Xu, 1999; Zhang et al., 2005).

Several studies have demonstrated that ozonated-water treatments promote shelf life extension of food products, and that ozonation is in fact an appropriate method to improve food quality and safety (Beltran et al., 2005; Graham, 2000; Manousaridis et al., 2005; Ölmez and Akbas, 2009). However, ozone decomposition is rapid in water and, consequently, its antimicrobial action may take place only at food surface (Aguayo et al., 2006). This action could be important if the goal is food surfaces decontamination, particularly for fruits and vegetables proper washings.

The main objective of this work was to study the impact of ozone in aqueous solution on the following microbial loads: *Listeria innocua* (in red bell peppers), total mesophiles (in strawberries) and total coliforms (in watercress) at two ozone concentrations. These combinations were used as case studies. Comparisons between the effectiveness of ozone treatments and conventional heat blanching, in the range temperature 50–60 °C, were also a target. Ozone treatments were combined with heat blanching with the purpose of studying possible synergistic effects and finding if the application of hurdle technologies would be more effective than the processes applied individually.

2. Materials and methods

2.1. Experimental procedures

2.1.1. *L. innocua* cultures

L. innocua NCTC 10528, obtained from Leatherhead Food Research Association (Leatherhead, UK), was subcultured (30 °C, 24 h) in Tryptic Soy Broth – TSB (Lab M, Lancashire, UK) containing 0.6% yeast extract – TSBYE (Lab M, Lancashire, UK). The cultures were maintained at 7 °C on Tryptic Soy Agar – TSA (Lab M, Lancashire, UK) supplemented with 0.6% yeast extract – TSAYE. Stationary phase culture of the bacterium was used in this study.

2.1.2. Fruits and vegetables samples

Strawberries (*Fragaria ananassa* D.), watercress (*Nasturtium officinale* R.Br.) and red bell peppers (*Capsicum annuum* L.) were purchased in a local market. The red bell peppers were pre-washed in deionised water and dried with absorbent paper. Peduncles and seeds were removed and samples were cut in small portions of approximately 20 g. Each sample was artificially inoculated at the internal surface with 250 µL (5 drops of 50 µL each) of the second *L. innocua* subculture (see Section 2.1.1), with a contact time of 15 min (until the surface had dried). Initial loads averaged 1.9×10^7 cfu/g (45 replicates). Strawberries and watercress were not washed and were not inoculated. Native total mesophiles (initial load averaged 5.6×10^7 cfu/g; 45 replicates) and total coliforms (initial load averaged 3.9×10^8 cfu/g; 47 replicates) were evaluated on strawberry and watercress samples, respectively.

2.1.3. Treatments

2.1.3.1. Ozonation. Ozone treatments were performed in a pilot plant. Oxygen was passed through a corona discharge generator (OZ5, SPO₃ – Sociedade Portuguesa de Ozono, Lda., Porto, Portugal) to produce ozone at 5 g/h. The generator was interconnected to a container filled with deionised water (approximate volume of 30 L; the ratio between mass of the samples and volume of

ozonated water was approximately 80 g/30 L), forming a closed circuit ring apparatus. Ozone was continuously incorporated by bubbling in the water (at ~15 °C) and the aqueous ozone concentration was measured by potential difference (Redox probe; SZ 275, B&C Electronics, Carnate, Italy).

The ozone generator operated at two different production capacities, resulting in two different concentrations of dissolved O₃ (0.3 and 2.0 ppm). These concentrations were maintained constant throughout treatment, and were confirmed using an ozone determination kit for O₃ in water (25180-50 Ozone AccuVac colour disc kit, HACH Lange GmbH, Düsseldorf, Germany).

Samples were immersed in ozonated water and the treatment times were 1, 2 and 3 min (experiments were carried out independently). At least three replicates of each treatment were done.

2.1.3.2. Blanching. Blanching treatments were performed in a crystallising basin containing 1 L of deionised water (the ratio between mass of the samples and volume of water was approximately 40 g/L). During blanching, water was stirred for temperature homogenisation and for simulation of the turbulent flow that occurred in the ozonated water tank. For each combination microorganism/food, different temperatures were tested as a result of different thermal sensitivities of the microorganisms under study. For *L. innocua*/red bell peppers, blanching temperatures were 50 and 60 °C; for total mesophiles/strawberries, the temperatures were 50 and 55 °C; for total coliforms/watercress, the temperatures were 50 and 55 °C. After blanching, samples were transferred to sterilized bags and placed in a mixture of ice-water.

The treatment times were 1, 2 and 3 min (experiments were carried out independently). At least three replicates of each treatment were done.

2.1.3.3. Combining ozonation and blanching. The following combined treatments were performed: (i) ozonation (0.3 ppm; 2 min) followed by blanching (1 min); (ii) blanching (1 min) followed by ozonation (0.3 ppm; 2 min).

The blanching temperatures were 50 and 60 °C for *L. innocua*/red bell peppers, and 50 and 55 °C for total mesophiles/strawberries and total coliforms/watercress. The temperature of 60 °C was not selected for total mesophiles and total coliforms, since preliminary experiments showed that at this temperature the microbial loads decreased till undetectable levels. *L. innocua* was more temperature resistant and consequently higher temperatures could be considered for combined treatments. At least three replicates of each treatment combination were done.

2.1.3.4. Control treatments. Experiments, as described in Section 2.1.3.1, were done in water alone (i.e. control of the ozonation treatment).

Water washings were performed as described in Section 2.1.3.2, but at a temperature of approximately 15 °C (control of the blanching treatment).

At least three replicates of each treatment were done.

2.1.4. Enumeration

Before and after each treatment, samples were aseptically cut in small pieces and homogenised in a stomacher using 80 mL of Buffered Peptone Water – BPW (Lab M, Lancashire, UK), for 5 min. Decimal dilutions were carried out in BPW.

L. innocua populations were assessed, in duplicate, using Palcam agar containing selective supplement (Merck, Darmstadt, Germany). Samples were incubated at 30 °C during 3 days (according to ISO 11290-1).

Total mesophiles populations were assessed, in duplicate, using Plate Count Agar – PCA (Lab M, Lancashire, UK). Samples were

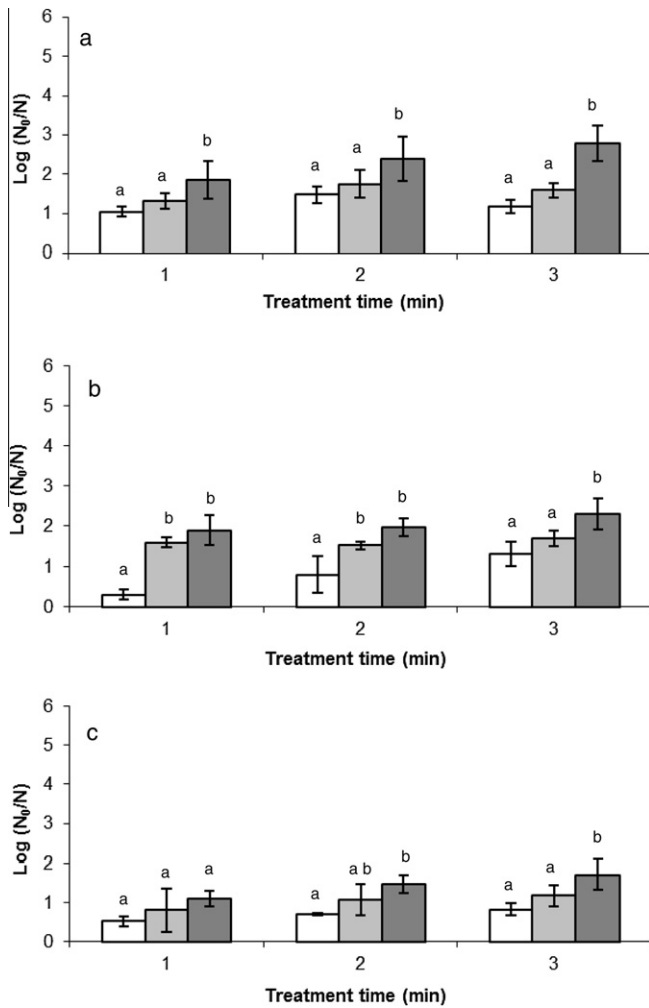


Fig. 1. Effect of water-washings (□) and ozonated water washings at 0.3 ppm (▨) and at 2.0 ppm (■) on load log-reductions of: (a) *L. innocua*/red bell peppers (initial loads averaged 1.9×10^7 cfu/g), (b) total mesophiles/strawberries (initial loads averaged 5.6×10^7 cfu/g), and (c) total coliforms/watercress (initial loads averaged 3.9×10^8 cfu/g). The bars represent standard deviations. Values with different characters, for the same treatment time, differ significantly ($p < 0.05$).

incubated at 30 °C during 3 days (according to ISO 4833:1991 and ISO 7218:1996).

Total coliforms populations were assessed by duplicate, using Violet Red Bile Agar – VRBA (Lab M, Lancashire, UK). Samples were incubated at 30 °C during 1 day (according to ISO 4832:1991).

2.2. Data analysis

The treatments effects were assessed by calculating the microbial reduction expressed in terms of log-cycles (i.e. $\log(N_0/N)$, where N_0 is the sample initial microbial load and N the microbial load after treatment).

Results were compared by analysis of variance (two-way ANOVA, testing time and treatment effects) using SPSS® 17.0 for Windows® (2008 SPSS Inc., Chicago, USA). Post-hoc tests were performed, for paired means comparison (Tukey's test; Walpole and Myers, 1993).

3. Results and discussion

The impact of ozone treatment on *L. innocua* (in red bell peppers; initial loads $\sim 10^7$ cfu/g), total mesophiles (in

strawberries; initial loads $\sim 10^7$ cfu/g) and total coliforms (in watercress; initial loads $\sim 10^8$ cfu/g) can be observed in Fig. 1. Total coliforms were selected for watercress, since these microorganisms are contaminants of this vegetable. Total mesophiles were chosen for strawberries, as representative microorganisms mixture of a fruit. *L. innocua* was selected as an indicator microorganism. This bacterium is often used as a surrogate of the pathogenic *Listeria monocytogenes* (Margolles et al., 2000).

In relation to the combination *L. innocua*/red bell pepper (Fig. 1a), results showed that there is a significant ozone concentration effect on microbial reduction ($p < 0.05$). This means that higher reductions were obtained when ozonated water at 2.0 ppm was used. It was also found a significant treatment time effect ($p < 0.05$). For red bell peppers treated with ozonated water at 0.3 ppm it was observed a microbial reduction of 1.3 ± 0.2 , 1.8 ± 0.4 and 1.6 ± 0.2 log cycles for a treatment time of 1, 2 and 3 min, respectively. This treatment was equivalent to simple water washings, for all times considered. Changing ozone concentration to 2.0 ppm, the log-reductions were higher and equal to 1.9 ± 0.5 , 2.4 ± 0.6 and 2.8 ± 0.5 for 1, 2 and 3 min, respectively.

For the case total mesophiles/strawberries (Fig. 1b), ozone treatments at both concentrations were significantly different ($p < 0.05$) from water washings, if lower treatment times were considered (1 and 2 min). Higher microbial reductions were attained for the highest ozone concentration and for 3 min (2.3 ± 0.4 log-cycles reduction). Treatment time impacts on microbial reductions were also significant ($p < 0.05$).

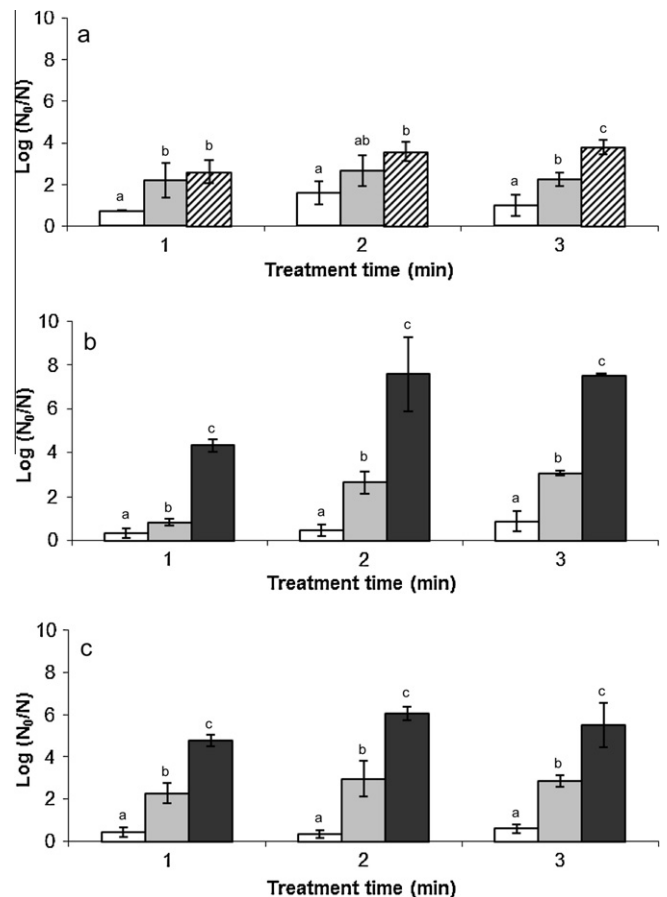


Fig. 2. Effect of water-washings (□) and blanching (▨, 50 °C; ■, 55 °C; ▩, 60 °C) on load log-reductions of: (a) *L. innocua*/red bell peppers, (b) total mesophiles/strawberries, and (c) total coliforms/watercress. The bars represent standard deviations.

Table 1
Effect of blanching at 50 °C and ozonated water at 2.0 ppm on microbial reductions of *L. innocua*/red bell peppers, total mesophiles/strawberries and total coliforms/watercress.

Time (min)	<i>L. innocua</i> red bell peppers		Total mesophiles strawberries		Total coliforms watercress	
	Blanching 50 °C	O ₃ 2 ppm	Blanching 50 °C	O ₃ 2 ppm	Blanching 50 °C	O ₃ 2 ppm
1	2.20 ± 0.81 ^a	1.85 ± 0.48 ^a	0.84 ± 0.15 ^a	1.89 ± 0.37 ^a	2.28 ± 0.47 ^a	1.11 ± 0.19 ^b
2	2.64 ± 0.75 ^a	2.39 ± 0.57 ^a	2.66 ± 0.51 ^a	1.96 ± 0.21 ^a	2.97 ± 0.84 ^a	1.47 ± 0.22 ^b
3	2.24 ± 0.31 ^a	2.78 ± 0.45 ^a	2.88 ± 0.12 ^a	2.31 ± 0.39 ^a	2.84 ± 0.2 ^a	1.71 ± 0.39 ^b

For each combination microorganism/food and for the same treatment time, values with different characters differ significantly ($p < 0.05$).

Significant time and treatment effects were also observed in total coliforms/watercress (Fig. 1c). Microbial log reductions obtained for ozonated water washings at 0.3 ppm were 0.8 ± 0.6 (1 min), 1.1 ± 0.4 (2 min) and 1.2 ± 0.3 (3 min). If ozone at 2.0 ppm was used, reductions attained 1.1 ± 0.2 (1 min), 1.5 ± 0.2 (2 min) and 1.7 ± 0.4 (3 min) log-cycles. The impact of ozone in reducing total coliforms load was significantly higher ($p < 0.05$) at the highest concentration and for 2 and 3 min of treatment.

The combination microorganism/food that presented higher microbial reductions was *L. innocua*/red bell pepper. This can be explained by the diversity of native flora enumerated in total mesophiles and total coliforms that may have different sensitivity to the ozone effect. The nature and composition of food surface, the degree of attachment to or association of microorganisms with food and biofilms formation are other possible justifications.

The effect of ozonated water-washings have been studied for several food products and microorganisms. The magnitude of the reported microbial reductions is comparable to the ones observed

in our study. However, it is often misled that these reductions are equivalent to simple water washings. This may have occurred in the microorganism/food studied.

Zhang et al. (2005) reported a decrease of 1.7 log-cycles of total microbial counts in fresh-cut celery treated with ozonated water (at 0.18 ppm). Beltran et al. (2005) used ozonated water (at 10, 20 and 10 ppm plus UV treatment) in lettuce washings, obtaining 1.6 and 3.2 log-reductions in aerobic mesophilic and coliforms counts, respectively. No significant concentration effect was detected. Akbas and Olmez (2007) reported a decrease of 1.7 and 1.5 log-cycles, respectively in mesophilic and psychrotrophic bacteria, for the same product (ozone at 4 ppm; 2 min). When applying ozonated water (3.85–3.95 ppm) in pre-cut green peppers washings, Ketteringham et al. (2006) observed log-reduction around 0.7. Yuk et al. (2007) reported that ozonated water treatments (up to 3 ppm of ozone concentration) applied to enoki mushrooms gave less than 1.0- and 0.5-log count reductions on *Escherichia coli* O157:H7 and *L. monocytogenes*, respectively.

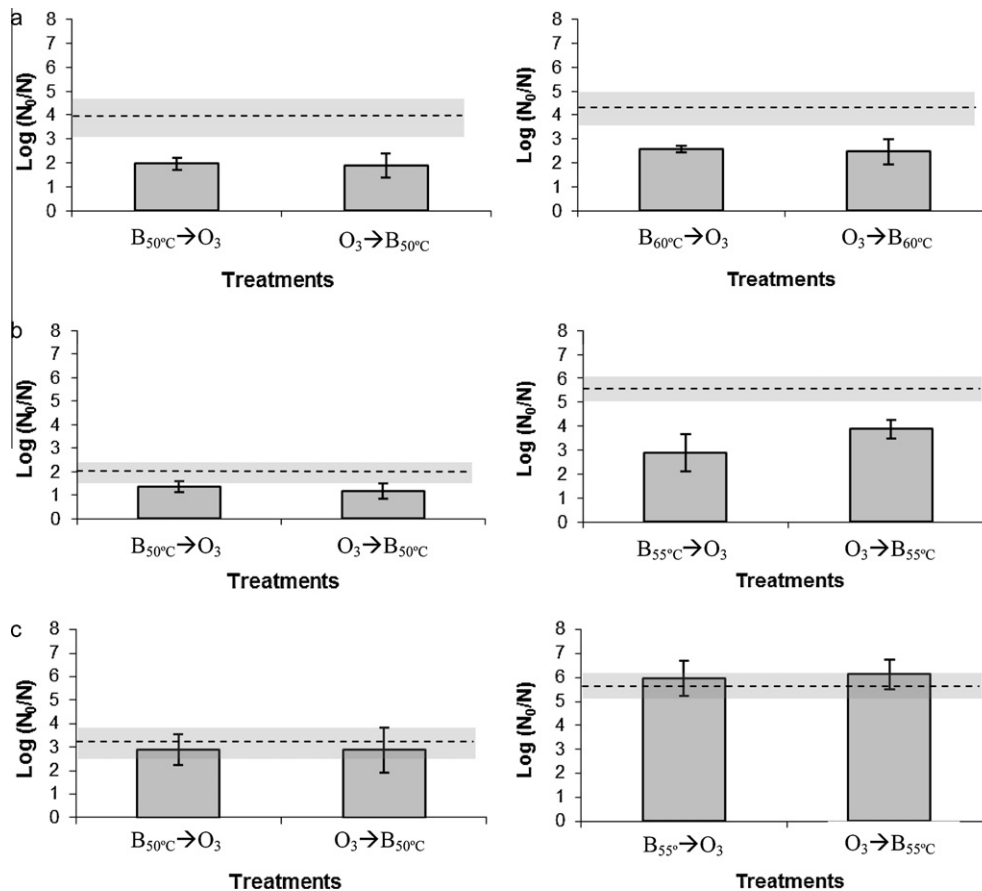


Fig. 3. Effect of combining blanching (B) with ozonated water washings at 0.3 ppm (O₃) on load log-reductions of: (a) *L. innocua*/red bell peppers, (b) total mesophiles/strawberries, and (c) total coliforms/watercress. The bars represent standard deviations of results obtained by combination of treatments. The dashed lines represent the additive effects of the treatments applied independently; the shaded areas include the limits of standard deviations.

This reduced ozone effectiveness (often comparable to water-washings) can be explained by the indiscriminate action of ozone to bacteria and organic matter. Food cut surfaces promote the leaching of organic matter and, consequently, ozone may react with these substances before acting on the bacteria present at the foods surfaces (Ketteringham et al., 2006; Khadre et al., 2001; Restaino et al., 1995; Suslow, 1998).

Further objectives of this work were to study improvements of the ozone impact, when the treatment was combined with blanching. This type of treatment, if efficient, can minimise the thermal impact of blanching on vegetables to be frozen. Consequently, blanching treatments were performed alone (aiming at concluding about the temperature effect) and results were compared to the ones obtained when blanching was combined with ozonation. The impact of mild blanching can be observed in Fig. 2. As expected, higher temperatures implied higher microbial reductions. However, total mesophiles were less temperature-resistant (Fig. 2b), when compared to total coliforms (Fig. 2c) and *L. innocua* (Fig. 2a).

When comparing results of blanching treatments *per se* with ozonated water washings, it is interesting to find that *L. innocua*/red bell peppers and total mesophiles/strawberries log-reductions at 50 °C were similar to the ones observed when ozone at the highest concentration and for same treatment times was used (results in Table 1). For total coliforms/watercress, blanching at 50 °C provided higher microbial reductions than ozone.

Ozone and blanching treatments were combined with the purpose of attaining synergistic interactions of greater magnitude than the sum of the effects obtained when treatments were individually applied. The order of the applied treatments was also tested. Results are in Fig. 3 (where the additive effects of individual treatments are also included). For some cases, the effects were additive: mesophiles/strawberries-combination of ozone and blanching at 50 °C (1.2 ± 0.3); coliforms/watercress-combination of ozone and blanching at 50 °C (2.9 ± 0.8) and 55 °C (6.0 ± 0.6). However, and for the remaining situations, antagonistic effects were observed. This means that the log-reductions obtained with combined treatments were lower than the sum of the effects when the treatments were applied independently and consequently a hurdle effect was not observed. Selma et al. (2008) arrived at similar results when studying the effect of gaseous ozone and hot water (75 °C/1 min) on microbial and sensory attributes of cantaloupe.

4. Conclusions

When ozone is applied in aqueous solution for reducing microbial loads in fruits and vegetables, its effectiveness is dependent on the combination microorganism/food. However, and for all cases studied, ozonated water-washings at the highest concentration were more effective than simple water-washings. The presence of ozone generally added an additional reduction of 0.5–1.0 log-cycles and can be considered an adequate sanitizer method for low-contaminated fruits and vegetables.

For the cases *L. innocua*/red bell peppers and total mesophiles/strawberries, the impact of ozonated water-washings in reducing microbial loads were similar to the ones obtained when a mild blanching at 50 °C was applied (for the same treatment time). For such cases, ozone could be a good alternative to the conventional heat blanching.

Additive and antagonistic effects were observed when ozonated water-washings were combined with blanching treatments. This means that, in some situations, the log-reductions obtained with combined treatments were equal to or lower than the sum of the effects when the treatments were applied individually.

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