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UV-C doses to reduce pathogen and spoilage bacterial growth *in vitro* and in baby spinach

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ABSTRACT

The aim of this work was to investigate the use of different doses of UV-C (0, 2.4, 7.2, 12 and 24 kJ m^{-2}) radiation treatments to inhibit microbial growth of *Listeria monocytogenes* and *Salmonella enterica*. The spoilage bacteria *Pseudomonas marginalis* (gram negative) was also tested. These bacteria were studied under *in vitro* conditions and in baby spinach leaves (*in vivo* conditions) for 13 and 14 d at 5 °C, respectively. All radiation doses were effective in reducing bacterial growth, although contrary to expectations, high doses did not show the highest microbial inhibition in *in vitro* experiments. UV-C doses (2.4–24 kJ m^{-2}) were also used on baby spinach (*Spinacia oleracea* L.) leaf surfaces, stored under humidified air using perforated plastic film. A clear inhibitory UV-C effect was observed on *L. monocytogenes* for 14 d at 5 °C. Meanwhile, UV-C radiation reduced *S. enterica* loads until the first 4 d of storage, after which a significant increase was found on radiated leaves compared to the control. *P. marginalis* counts were slightly reduced in UV-C treated leaves. In addition, significant decreases in psychrotrophic counts and *Enterobacteriaceae* were found during the first 4 d of storage. Respiration rates of baby spinach leaves were higher in radiated than in non-radiated leaves. Moreover, no obvious damage on the epidermal surface and to cell shape was detected in radiated and non-radiated leaves by scanning electronic microscopy (SEM). In summary, the use of double-sided UV-C radiation, at low doses, was effective in reducing initial microbial counts of the tested bacteria types and psychrotrophic and *Enterobacteria* counts, and in keeping *L. monocytogenes* at low levels during the storage period, without affecting the sensory quality of fresh-cut baby spinach leaves.

1. Introduction

Fresh-cut vegetables are very popular for consumption due to their convenience and ready-to-use properties, but they provide an ideal medium for microbial growth due to tissue damage caused by cutting. However, most such material is less contaminated than the original whole vegetable, as they are washed and disinfected (Nguyen-The and Carlin, 1994). Leafy vegetables are highly susceptible to mechanical damage, and bacterial and mould growth which drastically reduces shelf-life (Cantwell and Kasmire, 2002). According to Nguyen-The and Carlin (1994), fresh-cut products usually contain microorganisms such as psychrotrophic aerobic bacteria, fecal coliforms, yeast, moulds and pectinolytic bacteria.

Spinach leaves can harbor high numbers of mesophilic and psychrotrophic aerobic bacteria, identified mainly as pectinolytic species, such as *Pseudomonas* which is the major spoilage agent (Babic and Watada, 1996). During cold storage of fresh-cut leaf vegetables, pectinolytic strains of *Pseudomonas* are responsible for bacterial soft rot (Ahvenainen, 1996). As well as spoilage microorganisms, fresh-cut vegetables could harbor pathogens, among which *Listeria* is of great concern.

Listeriosis, caused by *Listeria monocytogenes*, is of great public health concern due to its clinical severity and high mortality rate, particularly in pregnant women, newborns, the elderly and the immunocompromised people (McLauchlin et al., 2004). *L. monocytogenes* is widely distributed in the environment and it is therefore present in a wide variety of raw food materials (Ryser and Marth, 1991). Pre-packaged mixed salads have a high potential for contamination by *L. monocytogenes* due to extensive handling during preparation, or by cross-contamination from the environment. The lack of any heating step prior to the consumption of such products places the emphasis on high-quality ingredients, hygienic manufacture, appropriate shelf-life, and correct storage for maintaining product safety (Little et al., 2007). Modified atmospheres have to be applied together with other preservative techniques in order to ensure the inhibition of *Listeria innocua* in fresh-cut vegetables

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(Scifo et al., 2009). *L. monocytogenes*, as well as *Salmonella* spp., may still survive and even proliferate at low temperatures (Ahvenainen, 1996).

In order to decrease microbial contamination, the fresh-cut industry commonly uses NaOCl and acids as disinfection agents but by-products such as trihalometanes and chloramines are potentially harmful for humans, so alternative disinfectant agents must be studied. One of these disinfectant agents is ultraviolet-C (UV-C) light, which occupies a wavelength band (200–280 nm) in the non-ionising region of the electromagnetic spectrum and the equipment for which is relatively inexpensive. The technique is subject to certain safety precautions, but is easy to use and the radiation is lethal to most types of microorganisms (Bintsis et al., 2000). It has been reported that 0.5–20 kJ m^{-2} UV-C doses inhibit microbial growth by inducing the formation of pyrimidine dimers which distort the DNA helix and block microbial cell replication. So, the cells become unable to repair their radiation-damaged DNA and die. There are, however, repair systems such as UV-induced enzymatic photorepair and expression of excision-repair genes that may restore DNA integrity in exposed microbial cells. The effectiveness of UV-C seems to be temperature independent (in the range from 5 to 37 °C), but depends on the irradiation incidence determined by the structure and topography of the surface of treated produce (Bintsis et al., 2000; Gardner and Shama, 2000), the fluence (J m^{-2}) and the position between the source and the sample. In addition, UV-C light acts indirectly against microorganisms by stimulating defence mechanisms in treated produce, delaying decay and senescence. The crucial point is whether a safe dose can be found which would greatly impair pathogen growth without damaging the product (Ben-Yehoshua and Mercier, 2005).

The combined use of MAP and UV-C radiation reduced psychrotrophic, coliform bacteria and yeast growth in fresh-cut 'Red Oak Leaf' lettuce, without adversely affecting sensory quality (Allende and Artés, 2003a). Other studies have reported that UV-C inhibited microbial growth, retarding decay and senescence of zucchini squash (Erkan et al., 2001), tomatoes (Liu et al., 1993; Lu et al., 1987), strawberries (Marquenie et al., 2002), carrots (Mercier and Arul, 1993), table grapes (Nigro et al., 1998) and sweet-potato (Stevens et al., 1999). However, UV-C can change the cell permeability in leafy vegetables, increasing the leakage of electrolytes, amino acids, and carbohydrates. This in turn can stimulate bacterial growth, which is inappropriate for improving shelf-life of fresh-cut products (Sztejnberg and Blakeman, 1973 cited in Nigro et al., 1998). Tomato fruit radiated with 3.7 kJ m^{-2} were more susceptible to disease caused by grey mould rot immediately after treatment, becoming gradually more resistant until 35 d at 13 °C (Charles et al., 2008a).

The purpose of this work was to evaluate the effects of different doses of UV-C radiation on pathogens and spoilage bacteria counts *in vitro* (selective media) and *in vivo* conditions (fresh-cut baby spinach leaves). In addition, the antimicrobial potential effect of UV-C radiation to reduce the growth of different bacterial strains, such as *L. monocytogenes* and *Salmonella enterica*, which naturally grow in fresh-cut salad was evaluated, since they are considered important for the food industry. On the other hand, the spoilage bacteria *Pseudomonas marginalis* was chosen, as this lives in soil and is in contact with vegetables which grow close to ground level, and can spoil the fresh appearance.

2. Materials and methods

2.1. Microorganisms and incubation conditions

Bacterial strains were obtained from the Spanish Type Culture Collection (CECT, http://www.cect.org). The selected media and the incubation conditions for each type of microorganism are shown in Table 1, according to the recommendations of CECT.

Bacteria were stored at −80 °C in vials of 1 mL glycerine solution:water (1:3). Two subsequent subcultures were grown in nutrient broth (50 mL) at 37 °C until the stationary phase for 24 h for each microorganism. From the second subculture a dilution series was made in sterile tryptone phosphate water (pH 7.0) (Scharlau Chemie S.A., Barcelona, Spain). Appropriate dilutions of 1 mL were poured in Petri dishes, and selective media to determine the microbial count were used in order to inoculate the samples (selected solid media in Petri dishes or baby spinach leaves) with an initial population density of 5–7 log cfu g^{-1}.

2.2. UV-C applications

The prototype UV-C table consisted of two blocks of 15 stainless-steel reflectors with unfiltered germicidal emitting lamps (254.7 nm, TUV 36W/G36 T8, Philips, Holland). A layer of bi-oriented polypropylene (BPP, 0.9 m × 1.5 m, 35-μm thick) was horizontally suspended in the middle of both the radiation blocks at 15 cm from each other. This film was placed over a steel frame supporting a polystyrene net that minimized the blockage of the UV-C radiation. The treatment chamber was covered with a protective reflecting inner layer, which enhanced homogeneous distribution of the emitted light and allowed indirect illumination of all sides. In order to determine the UV-C radiation intensity of the lamps and to verify the influence on blockage of the polystyrene net, a VLX 254 radiometer (Vilber Lourmat, Marne la Vallée, France) was used. The applied UV-C intensity was calculated as a mean of 12 UV-C readings on each side of the net with non-significant differences within both sides, which also shows any possible interference between UV-C and BPP film. The UV-C light intensity was kept constant, and the applied doses varied by altering the exposure time at the fixed distance (López-Rubira et al., 2005).

2.3. UV-C doses

The different UV-C doses applied were selected based on previous reports and our preliminary experiments (Allende and Artés, 2003a,b; Artés-Hernández et al., 2009), which determined the maximum radiation dose without detrimental effects on sensory quality of fresh leaf vegetables. The UV-C doses chosen were 0, 2.4, 7.2, 12, and 24 kJ m^{-2}. This latter dose, not tested before, was used as an extreme dose to determine the treatment effects on the microbial load, tissue damage and sensory quality.

2.4. In vitro experiment

One hundred microlitres (0.1 mL) of nutrient broth contaminated with *Listeria*, *Salmonella* and *Pseudomonas* was spread on solid selected media (Table 1) previously poured into Petri dishes, using disposable plastic spreaders under aseptic conditions. *In vitro* experiments were also carried out separately in order to analyze the effect of UV-C radiation on different microorganisms under optimal conditions (media and temperature, Table 1).

2.4.1. UV-C radiation

Inoculated Petri dishes were treated with different UV-C doses (0, 2.4, 7.2, 12, and 24 kJ m^{-2}) on the upper side where the bacteria were previously spread.

The UV-C treated dishes were placed in glass jars connected to a flushing panel with an air flow rate of 1 L h^{-1} with 95% RH and stored for 13 d at 5 °C in a cold room. Five inoculated dishes were made for each treatment and evaluation time.

Table 1
Media and incubation conditions selected for the different microorganisms.

Microorganism	Media	Incubation condition (time and temperature)
Listeria monocytogenes (CECT 4032)	Oxford agar base with a selective supplement (Scharlau Chemie, Barcelona, Spain)	2 d at 37 °C
Salmonella enterica subsp. *enterica* (CECT 4300)	Brilliant green agar (BGA, Scharlau Chemie, Barcelona, Spain)	2 d at 37 °C
Pseudomonas marginalis (CECT 229 T)	King B agar (F agar, Scharlau Chemie, Barcelona, Spain)	2 d at 30 °C

2.4.2. Microbial analyses

Five randomized Petri dishes were taken on days 0, 5, 9, and 13 to determine the microbial count under *in vitro* conditions. All the media content (about 20 g) in a dish was blended with the appropriated volume of sterile tryptone phosphate water (about 200 mL, pH 7.0) (Scharlau Chemie S.A., Barcelona, Spain) to obtain a 1:10 dilution in a sterile stomacher bag (Model 400 Bags 6141, London, UK) using a masticator (Seward Medical, London, UK). Serial dilutions were prepared in 9 mL tryptone phosphate water. From each dilution, 1 mL aliquots were aseptically pipetted for each bacteria microflora. The media and incubation conditions are shown in Table 1.

2.5. In vivo experiment

2.5.1. Plant material

Baby spinach leaves (*Spinacia oleracea* L. cv Silver Whale) were grown in the countryside of Cartagena (Murcia, Spain) and were mechanically harvested from December to February. Immediately after harvest, the leaves were transported in a portable ice box at 5 °C to the laboratory, where they were stored in a cold room at 5 °C. The next day, the leaves were processed in a disinfected room at 5 °C.

2.5.2. Sample preparation

Baby leaves with defects such as yellowing, decay, cuts and bruising were discarded. The selected leaves were washed by dipping for 1 min in tap water at 5 °C and then they were centrifuged for 45 s with a hand centrifuge (Dynamic Professional, Vence, France).

2.5.3. Inoculation of the baby spinach leaves

About 50 mL of the appropriated dilution (10^6–10^8 cfu 50 mL^{-1}) was sprayed on 1 kg of baby spinach leaves spread in a single layer to avoid superimposed leaves to obtain an initial count of 3–5 log cfu g^{-1}. The inoculation of the baby spinach leaves was performed in 2 experiments. In the first one, leaves were inoculated with *Listeria* and *Pseudomonas*. Later, in a second experiment, leaves were only inoculated with *Salmonella*.

2.5.4. UV-C radiation

About 500 g of inoculated baby spinach leaves were set on the BPP film, avoiding the overlapping of leaves in order to assure homogenous UV-C exposure. After the UV doses, 60 g of leaves was placed in polypropylene (PP) baskets of 1.5 mL (17 cm × 11.5 cm × 7.6 cm) and thermally sealed with an oriented PP film (OPP) of 35-μm thick (Plásticos del Segura, Murcia, Spain). Seven perforations were made with a 0.7-mm long needle on each basket to obtain humidified air condition (around 20% O_2, less than 0.3% CO_2 and ≥95% RH). Each basket corresponded to a replicate. Five baskets were prepared for each UV-C dose and evaluation time. Baskets were stored for 14 d at 5 °C.

2.5.5. Microbial analyses

To determine *Listeria* and *Pseudomonas* counts on fresh-cut spinach leaves, five randomized baskets were taken on days 0, 2, 5, 8, 12, and 14. For *Salmonella*, baskets were analyzed on days 0, 2, 5, 7, 9, and 14. A 10 g sample of leaves was blended with 90 mL of sterile tryptone phosphate water (pH 7.0) (Scharlau Chemie S.A., Barcelona, Spain) for 1 min in a sterile stomacher bag (Model 400 Bags 6141, London, UK) using a Masticator (Seward Medical, London, UK). Serial dilutions were prepared in 9 mL tryptone phosphate water. From each dilution, 1 mL aliquots were aseptically pipetted for bacteria microflora and 0.1 mL for yeasts and moulds. Incubation conditions for *Listeria*, *Pseudomonas* and *Salmonella* media are shown in Table 1.

Other non-inoculated (or naturally occurring) microorganisms (psychrotrophic bacteria, *Enterobacteria*, moulds and yeast) were also evaluated in both *in vivo* experiments, using the following media and incubation conditions: plate count modified agar (Scharlau Chemie S.A., Barcelona, Spain) for psychrotrophic aerobic bacteria, incubated at 7 °C for 7 d; violet red bile dextrose agar (VRBD, pH 7.2) (Scharlau Chemie S.A., Barcelona, Spain) for *Enterobacteria* incubated at 37 °C for 48 h; and potato dextrose agar base (Scharlau Chemie S.A., Barcelona, Spain) with the addition of oxytetracycline (100 mg L^{-1}) (Sigma Chemical Co., St. Louis, USA) for yeasts and moulds by spread, incubated for 2 and 5 d at 22 °C, respectively. All microbial counts were reported as log colony forming units per g of sample ($\log_{10}$ cfu g^{-1}).

2.6. Respiration rate

The possible physiological damage caused by the UV-C radiation was also studied, measuring the respiration rate as an expression of the metabolic activity of baby spinach leaves. Following the methodology explained in Sections 2.5.1, 2.5.2, and 2.5.4, samples of 150 g of UV-C treated leaves (without microbial inoculation) were put into 1.5 L glass jars. For each UV-C treatment, five jars were connected to a flushing panel with an air flow rate of 1 L h^{-1} with 95% RH. The jars were closed and the initial head space composition (O_2, CO_2 and N_2) was monitored using a 1 mL gas sample, which was injected into a gas chromatograph (Thermo Finnigan Trace GC, Milan, Italy) equipped with a thermal conductivity detector (150 °C), oven (from 40 to 90 °C), injector (150 °C) and with a Porapack-N 80/100 column and a Molsieve molecular sieve for CO_2 and O_2, respectively. Helium (He) was used as a carrier gas (20 mL min^{-1}). The headspaces were analyzed again 2 h later. The measurements were conducted periodically during the storage period. Between measurements, the jars were continuously flushed with humidified air. The respiration rate was expressed as CO_2 production (nmol kg^{-1} s^{-1}).

2.7. Scanning electron microscopy

Non-inoculated leaves were UV-C radiated following the same methodology described in Section 2.6. From each treatment, ten fresh and intact leaves were used and at least four pieces per leaf were used. Samples were radiated only on the upper side.

The leaf tissue was dehydrated in a graded acetone series (from 15 to 100%, v/v). Subsequently, the specimens were transferred to a critical point drying apparatus (CPD 030; BAL-TEC, AG; Balzers; Liechtenstein) and dried with CO_2. The dried specimens were mounted on aluminium discs and coated with gold in a Sputter Coater (SC7640, Quorum Technologies, East Sussex, England) to make their surfaces electrically conductive, and were finally

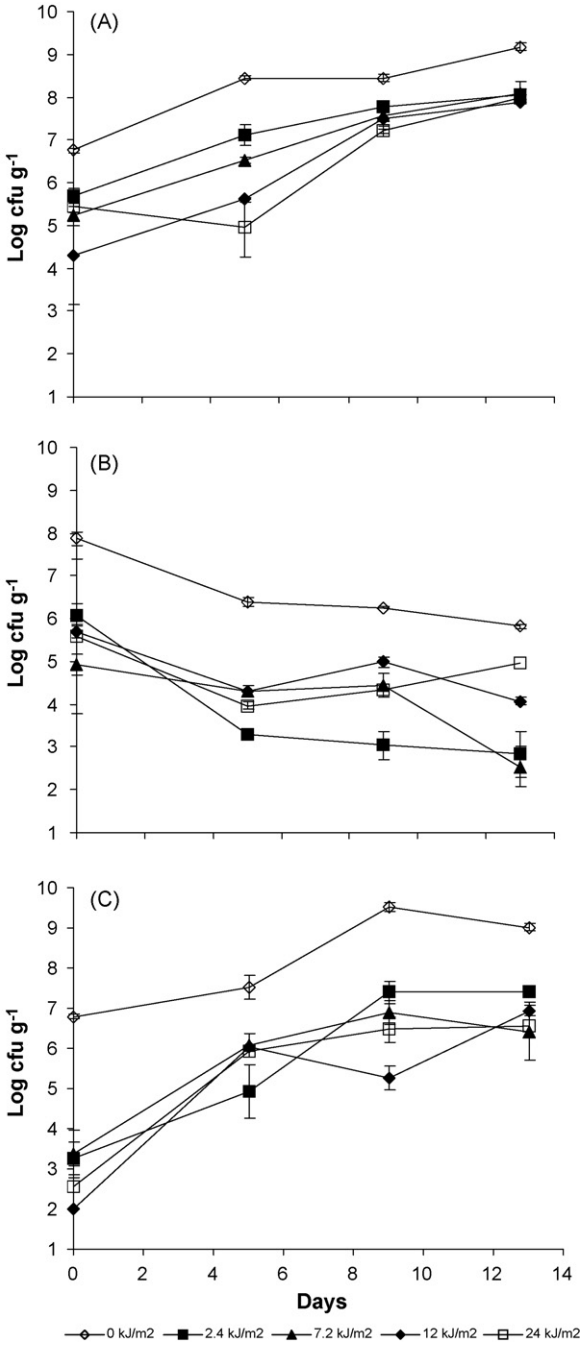

Fig. 1. Growth of inoculated bacteria in Petri dishes stored for 13 d at 5 °C. (A) *Listeria monocytogenes*; (B) *Salmonella enterica*; (C) *Pseudomonas marginalis*. Mean ($n = 5$) ± SE.

observed with a scanning electron microscope (S-3500N, Hitachi, Ratingen, Germany). Samples were taken from both *in vivo* experiments and inspected at 2000× of zoom.

3. Results and discussion

3.1. Microbial growth

3.1.1. In vitro conditions

3.1.1.1. Listeria monocytogenes and Salmonella enterica (pathogen bacteria). The initial *Listeria* and *Salmonella* counts significantly decreased by the application of UV-C radiation (2.4–24 kJ m^{-2}, Fig. 1A and B). The initial *Listeria* counts were 6.8 log cfu g^{-1} for control (0 kJ m^{-2}), while it was lower (4.3–5.7 log cfu g^{-1}) for UV-C radiated samples (Fig. 1A). Therefore, with UV-C radiation treatment, the initial *Listeria* and *Salmonella* counts were reduced by about 1–2.5 log cfu g^{-1} on the surface of dishes and it increased in all treatments at the end of storage at 5 °C (day 13). However, the control samples showed the highest *Salmonella* counts (9.2 log cfu g^{-1} vs. 7.9–8.1 log cfu g^{-1}, respectively) (Fig. 1A).

The samples treated with UV-C showed *Salmonella* counts around 4.9–6.1 log cfu g^{-1}, while it was higher in control samples (7.9 log cfu g^{-1}) at the beginning of the experiment (Fig. 1B). *Salmonella* counts diminished 4.0–5.0 log cfu g^{-1} for 12 and 24 kJ m^{-2}, respectively, after 13 d at 5 °C. However, low UV-C doses (2.4 and 7.2 kJ m^{-2}) showed a higher inhibitory effect on *Salmonella* counts (2.5–2.8 log cfu g^{-1}) compared to high UV-C doses. This finding was unexpected because high doses might have higher inhibitory effects.

Therefore, UV-C decreased *Listeria* and *Salmonella* counts at the beginning of storage, but the surviving bacteria later appeared to follow the behaviour of non-radiated bacteria.

3.1.1.2. Pseudomonas marginalis (spoilage bacteria). Pseudomonas was detected by its fluorescence under UV light, allowing the counting of inoculated bacteria (*in vitro* condition). UV-C treatments also showed an inhibitory effect on the *Pseudomonas* counts compared to the control (Fig. 1C). At day 0, the control treatment had 6.8 log cfu g^{-1} while in low UV-C doses (2.4 and 7.2 kJ m^{-2}) the counts decreased to 3.4 log cfu g^{-1}, and 2.0–2.5 log cfu g^{-1} when high UV-C doses (12 and 24 kJ m^{-2}) were used. During storage, *Pseudomonas* counts increased until the end of the experiment in all treatments, but remained lower than the control. Using 2.4 kJ m^{-2} radiation, the counts were slightly higher than other radiation treatments. After 13 d at 5 °C, the control reached 9.0 log cfu g^{-1} compared to 6.4–7.4 log cfu g^{-1} in radiated treatments (Fig. 1C).

Allende et al. (2006) reported that after 24 h in *in vitro* conditions, *Salmonella typhimurium* had completely failed to grow on plates treated with 0.085 kJ m^{-2}; but other strains, such as *Pseudomonas fluorescens*, only needed 0.03 kJ m^{-2} to inhibit growth. In our assays, the UV-C doses were higher than those applied by Allende et al. (2006), and while *Salmonella* counts did not show an increase during storage, bacterial counts were still found after 13 d. In addition, *Listeria* and *Pseudomonas* kept growing from the reduced initial counts after UV-C application. In general, UV-C doses higher than 2.4 kJ m^{-2} did not have a high inhibitory effect on bacterial growth. This unexpected response could be explained by the superficial bacterial effect of UV-C treatment. Baby leaves showed irregular topography and natural materials, such as wax and crystals, as shown by ESM (Fig. 6). These materials could serve as a protection, so the radiation was not so effective in these areas.

3.1.2. In vivo experiment

3.1.2.1. Listeria monocytogenes and Pseudomonas (experiment 1). An initial decrease in bacteria by applying UV-C on baby spinach leaves was observed (Fig. 2). As with the results obtained in *in vitro* conditions, the UV-C radiation reduced initial *Listeria* counts compared to the control (Fig. 2A). At day 0, UV-C treated and control leaves had 2.2–2.9 and 4.4 log cfu g^{-1}, respectively. During storage, the control reached 4.2–4.7 log cfu g^{-1}, while UV-C treated leaves increased slightly to 3.6–4.5 log cfu g^{-1}. After 14 d at 5 °C, leaves treated with 2.4–12 kJ m^{-2} had 3.6–4.2 log cfu g^{-1} although using 24 and 0 kJ m^{-2}, 4.5 and 4.6 log cfu g^{-1} were found, respectively. It is likely that the high dose of 24 kJ m^{-2} caused tissue damage, weakening the tissue. This condition allowed for higher counts in the UV-C treatment than the control at the end of the experiment. Other studies have also shown the inability of UV-C to inhibit or

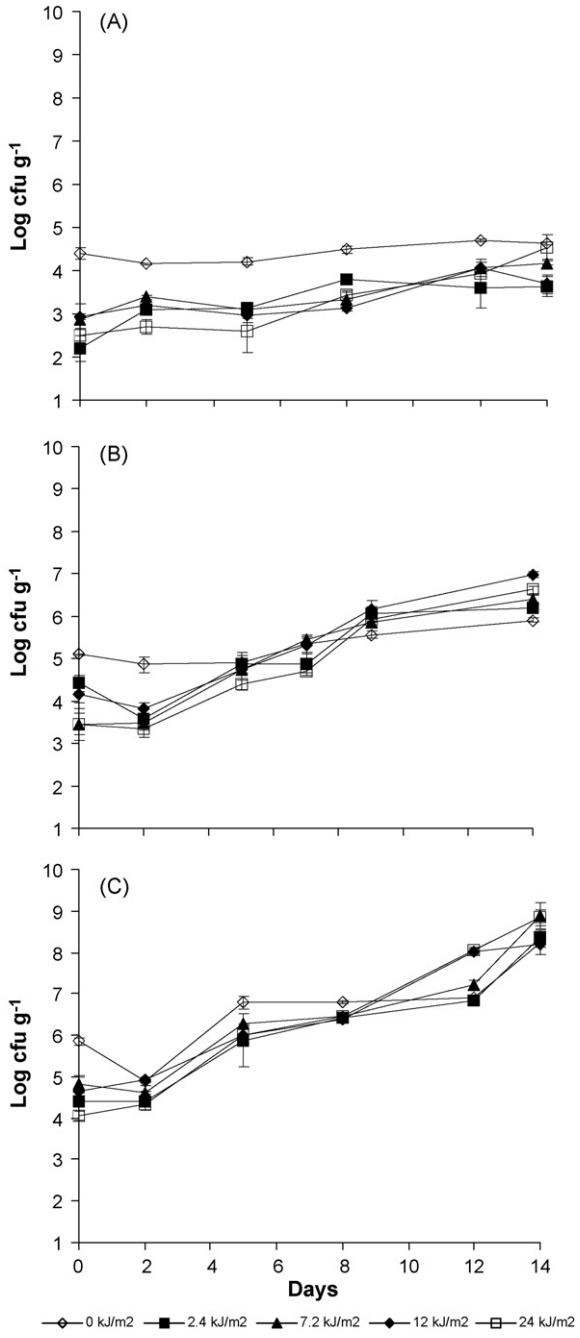

Fig. 2. Growth of inoculated bacteria in fresh-cut baby spinach leaves stored for 14 d at 5 °C. (A) *Listeria monocytogenes*; (B) *Salmonella enterica*; (C) *Pseudomonas marginalis*. Mean ($n = 5$) ± SE.

reduce pathogen growth on green leaf lettuce (Yaun et al., 2004) but the results were not related with tissue damage.

For *Pseudomonas*, a slight decrease in the initial count was observed using UV-C (Fig. 2B). At the beginning, control leaves reached 5.9 log cfu g^{-1} while leaves treated with 2.4–12 kJ m^{-2} had 4.4–4.8 log cfu g^{-1}. Meanwhile initial counts of 4.0 log cfu g^{-1} were obtained using 24 kJ m^{-2}. During storage, *Pseudomonas* counts increased in all treatments. At the end of storage, no clear differences were obtained among radiated and non-radiated treatments, reaching 8.2–8.9 log cfu g^{-1}, respectively. It is possible that the use of UV-C radiation on baby spinach leaves caused slight damage to the leaf surface, increasing the nutrient availability for the growth of *Pseudomonas* bacteria.

3.1.2.1.1. Other non-inoculated microorganisms (natural flora) in baby spinach (psychrotrophic bacteria, Enterobacteria, moulds and yeast). The initial psychrotrophic counts were 5.3 and 3.6–4.6 log cfu g^{-1} for control and UV-C treatments, respectively (Fig. 3A). The psychrotrophic bacteria increased throughout storage, reaching higher counts on UV-C treated leaves (7.6–8.7 log cfu g^{-1}) than control (6.8 log cfu g^{-1}) on day 14 (Fig. 3A). A similar trend was observed for *Enterobacteria* growth after the UV-C applications on baby spinach leaves (Fig. 3C). The initial control count (4.3 log cfu g^{-1}) was higher than on UV-C treated leaves (3.3–3.8 log cfu g^{-1}). However, after day 8, UV-C treated leaves had counts of 1–1.5 log cfu g^{-1} above those found in the control treatment. At the end of storage, UV-C treatments from 7.2 to 24 kJ m^{-2} reached 6.6–7.2 log cfu g^{-1}, and the control and 2.4 kJ m^{-2} had about 6.0 log cfu g^{-1} (Fig. 3C). Similar results were found for psychrotrophic bacteria and *Enterobacteria* during the second experiment (Fig. 3B and D). These results showed an inhibitory effect of UV-C radiation on initial microbial counts from baby spinach leaves. However, this effect was lost during storage, reaching higher counts on radiated leaves compared to the control. It is possible that the use of UV-C radiation on baby spinach leaves caused slight damage to the leaf surface, increasing the nutrient availability for growth of some bacteria.

Relatively low UV-C doses (4.54–11.35 kJ m^{-2}) showed no clear inhibitory effect on microbial growth in fresh-cut spinach stored at 5 °C (Artés-Hernández et al., 2009). These authors hypothesized that higher doses of UV-C would cause superficial damage on spinach leaves and could stimulate bacterial growth. Other studies have reported that UV-C inhibited microbial growth and delayed senescence of zucchini squash (Erkan et al., 2001), carrots (Mercier and Arul, 1993), or sweet-potato (Stevens et al., 1999). UV-C effectively decreased psychrotrophic bacteria, coliforms and yeasts growth in fresh-cut 'Red Oak Leaf' lettuce (Allende and Artés, 2003a; Allende et al., 2006). Allende and Artés (2003b) found a small reduction in psychrotrophic bacterial growth using 8.14 kJ m^{-2} in 'Lolo Rosso' lettuce stored in MAP at 5 °C. In that experiment, all the tested UV-C radiation reduced psychrotrophic growth by about 0.5–2 log cfu g^{-1} at the beginning of the storage. Maximum growth reductions of bacteria on 'Red Oak Leaf' lettuce were observed between 2 and 6 d of storage for 2.37 and 7.11 kJ m^{-2} (Allende et al., 2006). Erkan et al. (2001) reduced aerobic bacterial counts in sliced zucchini squash using 4.93 and 9.86 kJ m^{-2}. The effectiveness of UV-C depends on the incident irradiation, determined by the structure and topography of the surface of the product (Gardner and Shama, 2000). It is also known that the sensitivity of bacteria to UV-C varies with species and also among different strains of the same species (Block, 1977). Several bacteria and yeast have a potent repair mechanism of photo-reactivation. The exposure of cells to visible light after UV-C treatment induces enzymatic photo-repair and expression of excision-repair genes that may restore DNA integrity (Sommer et al., 2000; Lado and Yousef, 2002). Therefore, by this repair mechanism, they recover viability following UV radiation (Mercier et al., 2000).

The mould growth was slightly affected by UV-C radiation (Fig. 4A). At day 0, the mould counts were reduced more at higher UV-C doses. After 14 d at 5 °C, the mould counts ranged between 3.2 and 3.6 log cfu g^{-1} for all treatments. A similar effect of UV-C on initial yeast count was also detected (Fig. 4C). UV-C doses of 12–24 kJ m^{-2} reduced the initial yeast count to 2.5–3.1 log cfu g^{-1} compared to the control (4.1 log cfu g^{-1}). After 14 d, the yeast counts increased for UV-C treated leaves, reaching 5.5–5.7 log cfu g^{-1}, while in the control the count was 5.0 log cfu g^{-1}. Nigro et al. (1998) reported that grey mould was reduced in table grapes using very low UV-C doses (0.125–0.5 kJ m^{-2}). In fact, higher UV-C doses resulted in an increasing number of infected berries and lesion diameter.

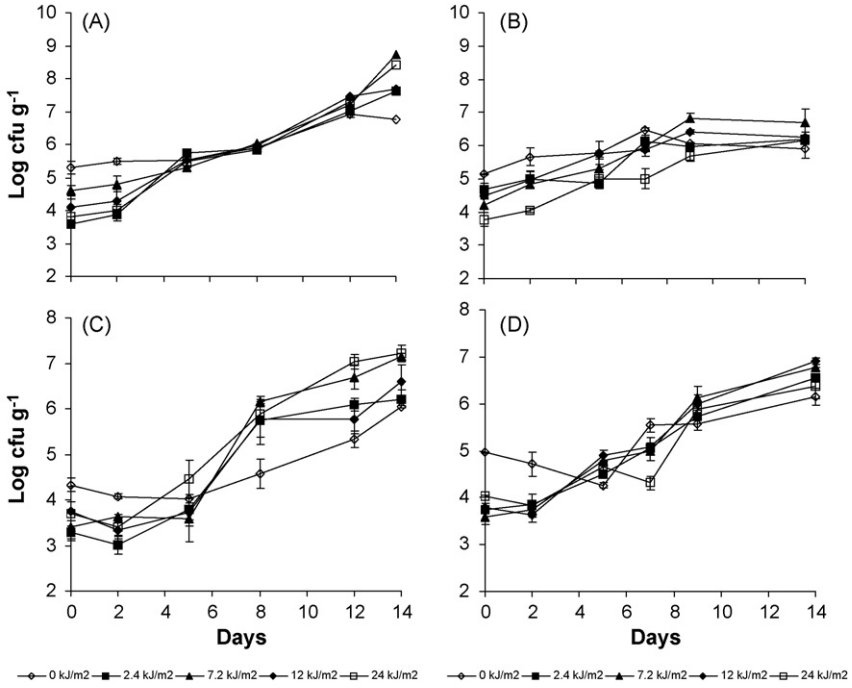

Fig. 3. Microbial growth in fresh-cut baby spinach leaves stored for 14 d at 5 °C. (A) and (B) psychrotrophic bacteria counts correspond to experiments 1 and 2, respectively; (C) and (D) *Enterobacteria* growth correspond to experiments 1 and 2, respectively. Mean ($n = 5$) ± SE.

3.1.2.2. Salmonella experiment (experiment 2). A clear decrease in *Salmonella* count was detected using UV-C on day 0 (Fig. 2B). Inoculated baby spinach leaves radiated with 2.4–24 kJ m^{-2} yielded 3.5–4.4 log cfu g^{-1} in comparison with 5.1 log cfu g^{-1} for the control. An increase in *Salmonella* growth to 5.9 log cfu g^{-1} for non-radiated leaves after 14 d was found (Fig. 2C). At the same day, radiated samples ranged from 6.2 to 7.0 log cfu g^{-1}. Therefore, UV-C decreased *Salmonella* counts during the first days of storage. During the course of the experiment, UV-C treated leaves showed higher microbial counts than control, possibly due to slight tissue damage and less competition with other bacteria groups, which might allow a faster growing rate of bacteria. As with our results, Yaun et al. (2004) found no ability of UV-C to inactivate a higher population of *Salmonella* spp. on the surface of green leaf lettuce but they did not discuss this response. However, Zhaung et al. (1995) reported that UV-C was more effective for reducing *Salmonella* on tomatoes than 320 mg L^{-1} chlorine.

3.1.2.2.1. Other non-inoculated microorganisms (natural flora) in baby spinach (psychrotrophic bacteria, Enterobacteria, mould and yeast). In this experiment, the initial psychrotrophic counts for

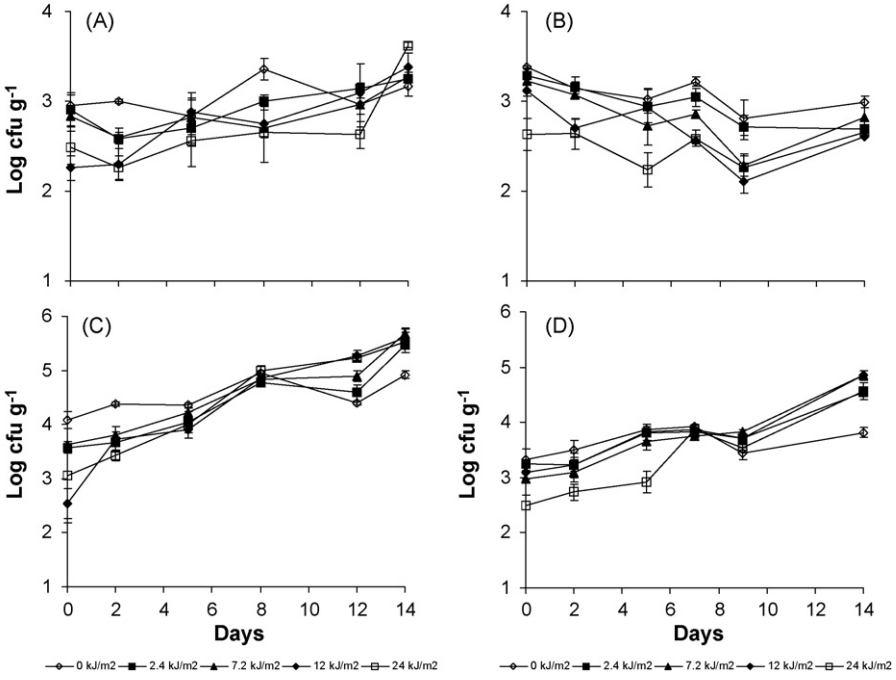

Fig. 4. Microbial growth in fresh-cut baby spinach leaves stored for 14 d at 5 °C. (A) and (B) mould counts correspond to experiments 1 and 2, respectively; (C) and (D) yeast counts correspond to experiments 1 and 2, respectively. Mean ($n = 5$) ± SE.

control and radiated leaves were around 5.2 and 3.8–4.7 log cfu g^{-1}, respectively (Fig. 3B). At 24 kJ m^{-2}, a decrease of 2 log cfu g^{-1} was detected in psychrotrophic counts, compared to non-radiated leaves. Up to 7 d at 5 °C, lower counts were found in radiated than in control leaves. Nevertheless, increased psychrotrophic counts were recovered after 14 d in radiated treatments, especially at high UV-C doses.

Initial *Enterobacteria* were reduced by UV-C (Fig. 3D) as similar to the previous *in vivo* experiment (Fig. 3C). Initial counts of 3.6–4.0 and 5.0 log cfu g^{-1} were found for radiated and control samples, respectively. On day 7, lower *Enterobacteria* growth was found in UV-C treatments than in the control. After 14 d, radiated and control samples had 6.4–6.9 and 6.2 log cfu g^{-1}, respectively. Therefore, UV-C can reduce the initial counts, which could be considered as a good alternative to chlorine during the processing line before packaging. However, a high bacterial growth trend was found in UV-C radiated leaves after 9 d at 5 °C, confirming previous results reported by Artés-Hernández et al. (2009) for psychrotrophics and *Enterobacteria*.

A similar mould growth trend was found in comparison to the previous *in vivo* experiment (Fig. 4A and B). A small inhibitory effect of high UV-C dose on mould and yeast growth was detected, reducing counts by 0.5–1 log cfu g^{-1} compared to control (Fig. 4B and D). In addition, erratic mould growth was found on baby spinach leaves during storage. In contrast, little increase in yeast growth was found in control treatments during storage (Fig. 4D). Higher yeast counts were detected in radiated samples (4.5–4.8 log cfu g^{-1}) compared to the control (3.8 log cfu g^{-1}) on day 14.

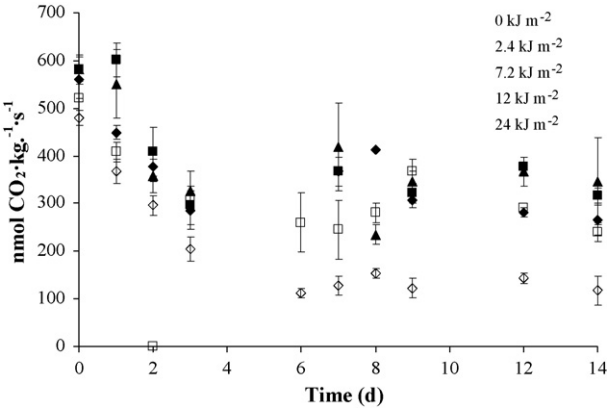

Fig. 5. Respiration rate (nmol CO_2 kg^{-1} s^{-1}) of baby spinach leaves treated with different UV-C doses (0–24 kJ m^{-2}) and stored for 14 d at 5 °C under humidified air. Mean (n = 5) ± SE.

3.2. Respiration rate

The respiration rate was high at the beginning and declined during storage for all the treatments (Fig. 5). Two hours after processing, the respiration was 480 nmol CO_2 kg^{-1} s^{-1} for non-UV-C radiated and 520–582 nmol CO_2 kg^{-1} s^{-1} for UV-C radiated leaves (Fig. 5). These high respiration rates obtained at the beginning could be explained by the tissue stress probably caused by the processing. After 3 d at 5 °C, steady rates were found

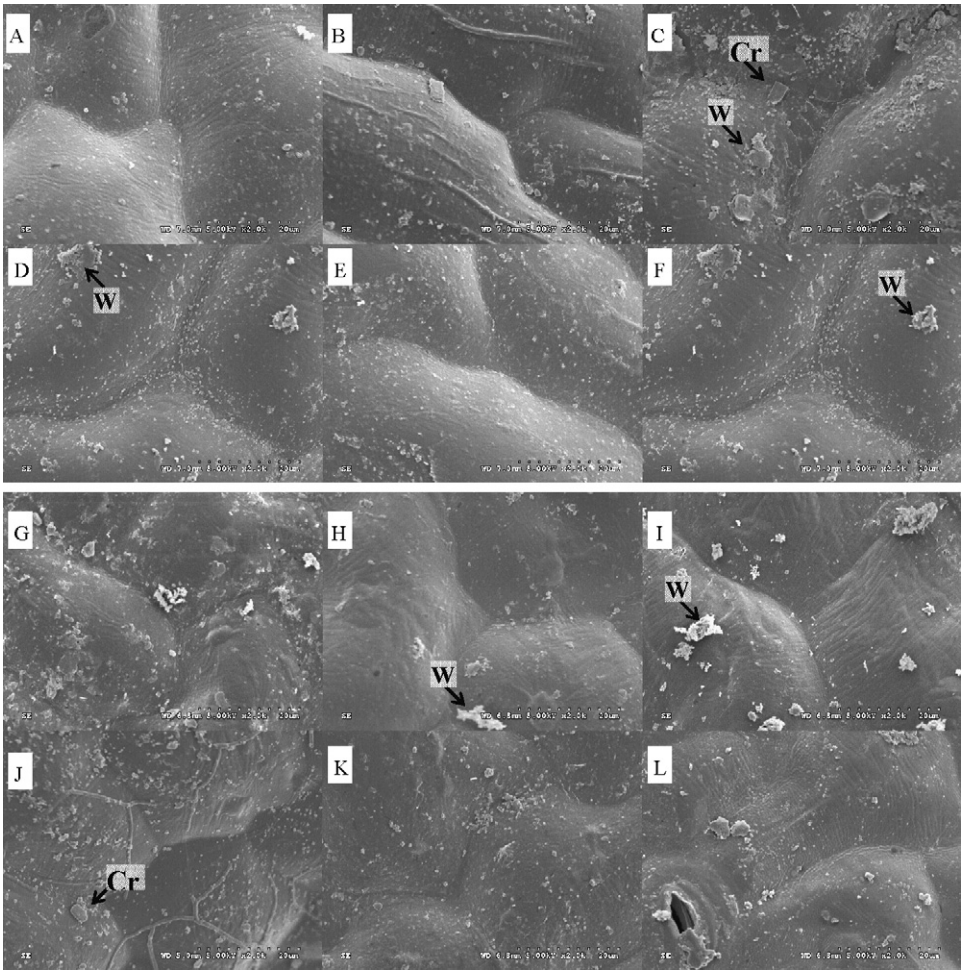

Fig. 6. Images of upper surface of baby spinach leaves treated with 0 kJ m^{-2} (A–F) and 24 kJ m^{-2} (G–L) and, immediately dried with CO_2 on day 0. These images correspond to the first experiment. W: epicuticular wax and Cr: crystal prism.

for all treatments, being two- or three-fold lower in the control (112–153 nmol CO_2 kg^{-1} s^{-1}) compared to radiated leaves (234–419 nmol CO_2 kg^{-1} s^{-1}). This could be explained by slight tissue damage caused by UV-C. However, no significant differences were found among UV-C treatments (2.4–24 kJ m^{-2}). This result was not expected, because higher UV-C doses usually cause higher respiration rates, especially when the dose is increased by 10-fold. For extending the fresh quality and reducing microbial growth of fresh-cut 'Red Oak Leaf' lettuce, UV-C treatments of 0.4, 0.81, 2.44, 4.07, and 8.14 kJ m^{-2} were applied (Allende and Artés, 2003a). According to this study, increased respiration and CO_2 levels within MAP were reported with an increase in UV-C intensities. However, radiated lettuce with 4.07 and 8.14 kJ m^{-2} showed similar respiration rates of 551–563 nmol CO_2 kg^{-1} s^{-1} during 5 d at 5 °C in air (Allende and Artés, 2003a). These results agree with previous reports on lettuce, tomato and zucchini squash where UV-C raised respiration (Maharaj et al., 1999; Erkan et al., 2001). It is possible that cells are damaged by UV-C light, and this is related with a respiratory stress in fresh-cut spinach. These results contradicted those found in UV-C treated broccoli with doses from 4 to 14 kJ m^{-2}, where all treatments reduced the respiration rate and delayed tissue damage (Costa et al., 2006). Therefore, the responses to UV-C stress would depend on several factors such as type of product, shape, surface to volume ratio, surface topography, etc., and this must be studied for each product and proposed situation.

3.3. Scanned images of the baby spinach leaves

Non-radiated and radiated leaves showed no obvious damage on the epidermis surface and the cell shape (Fig. 6). Similar results were found in the second experiment. In Fig. 6, only images from the first experiment *in vivo* (0 and 24 kJ m^{-2}) are shown. The results obtained in the second experiment were similar and no differences were found among treatments (data not shown). Therefore, according to the images taken by electron microscopy, UV-C doses from 0 to 24 kJ m^{-2} did not cause any visual damage on the surface tissue of baby spinach leaves. The surface of control and treated leaves was characterized by the presence of an amorphous epicuticular wax, and prism-shaped crystals. This makes us think that these deposits could protect the surface from the incidence of UV-C light. In fact, Charles et al. (2008b) found that a reduced pathogen colonization of UV-treated tomato fruit could not be attributed solely to changes in surface topography. They hypothesized that UV-C light (3.7 kJ m^{-2}) caused alteration in the amount of epicuticular wax and its ultra-structural arrangement in tomato fruit, presumably due to changes in its chemical composition. In another report, Charles et al. (2008c) said that after 3.7 kJ m^{-2}, tomato fruit induced decay resistance, caused by ultra-structural modifications of the pericarp tissue. UV-C induced plasmolysis of the epicarp cells, as well as some cell layers of the mesocarp, led to the formation of the cell wall stacking zone.

Thus the UV-C dose–response relationship can be biphasic, in which high doses are detrimental and lower doses stimulate beneficial reactions on the quality of baby spinach leaves. Therefore lower UV-C doses such as 2.4 kJ m^{-2} would be sufficient to delay microbial growth, avoiding potential tissue damage, with reduced energy costs (than higher UV-C doses) for extending the shelf-life of fresh-cut baby spinach.

Regarding the sensory analysis, there were no clear differences among the samples treated with different UV-C doses.

4. Conclusions

UV-C radiation was applied to both sides of baby spinach leaves in order to simulate a continuous production chain. The results showed effectiveness of initial microbial reductions in fresh-cut spinach at the beginning of storage using short exposure times and low radiation doses. Almost all the analyzed microbial groups were reduced by UV-C radiation throughout the storage period. UV-C light significantly reduced *L. monocytogenes* growth in fresh-cut spinach for 14 d at 5 °C. During the first 5–8 d, radiated leaves had lower *S. enterica* and *P. marginalis* counts compared to non-radiated samples. However these radiated leaves reached higher counts than control after 8 d of storage. A low UV-C dose (2.4 kJ m^{-2}) had a similar inhibitory influence on other microbial growth, compared to high doses such as 12 or 24 kJ m^{-2}. UV-C light was also effective at reducing psychrotrophic and *Enterobacteria* in fresh-cut spinach until 4 d at 5 °C. It is possible that UV-C light caused some tissue damage of the spinach leaves, as measured by an increase in respiration, but the surface tissue does not appear damaged when it was inspected by electron microscopy. Therefore, it can be concluded that UV-C radiation applied at proper doses and to both sides of the product could reduce microbial growth and extend shelf-life without adversely affecting the quality of fresh-cut baby spinach leaves.

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