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TESIS DOCTORAL

TRATAMIENTOS POSTCOSECHA INNOVADORES DE DESINFECCIÓN Y MANTENIMIENTO DE LA CALIDAD EN BROTES DE HORTALIZAS FOLIÁCEAS MÍNIMAMENTE PROCESADAS Y TOMATE

INNOVATIVE POSTHARVEST TREATMENTS FOR SANITATION AND KEEPING QUALITY IN FRESH-CUT BABY LEAVES AND WHOLE TOMATO

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La presentación de la Tesis Doctoral titulada "TRATAMIENTOS POSTCOSECHA INNOVADORES DE DESINFECCIÓN Y MANTENIMIENTO DE LA CALIDAD EN BROTES DE HORTALIZAS FOLIÁCEAS MÍNIMAMENTE PROCESADAS Y TOMATE" realizada por D. Alejandro Tomás Callejas, bajo nuestra dirección y supervisión, en el Departamento de Ingeniería de los Alimentos y del Equipamiento Agrícola, y que presenta para la obtención del grado de Doctor por la Universidad Politécnica de Cartagena.

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LOS DIRECTORES DE TESIS

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A mis padres y mis hermanos

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RESUMEN

Los avances científicos y tecnológicos de las últimas décadas han permitido conocer el beneficio para la salud del consumo de frutas y hortalizas. Diversos estudios epidemiológicos han demostrado la asociación entre la ingesta de frutas y hortalizas, con alto contenido en compuestos antioxidantes, y la prevención de ciertos tipos de cáncer y enfermedades cardiovasculares. Además, el consumo ha sido promovido por investigadores, nutricionistas e incluso a nivel gubernamental, recomendándose al menos cinco raciones de frutas y hortalizas en la dieta diaria.

El actual ritmo de vida, en el que cada vez hay menos tiempo para la preparación de una comida equilibrada, ha propiciado la aparición de un nuevo tipo de consumidor que demanda productos frescos, saludables y listos para consumir. Los vegetales mínimamente procesados en fresco (MPF), o de la "IV Gama" de la alimentación, son productos frescos acondicionados para su consumo directo, que se comercializan refrigerados y envasados en atmósfera modificada. Los tratamientos que sufren en su preparación industrial producen cambios poco notables respecto al vegetal original, en las propiedades deseables para el alimento y, en particular, de las nutritivas, organolépticas y de las relacionadas con su facilidad de utilización o conveniencia.

En los últimos años ha aumentado la aparición de brotes de contaminaciones alimentarias de origen microbiano asociadas a los productos vegetales enteros y MPF y, consecuentemente, también la preocupación por la calidad y seguridad de este tipo de productos por parte de los consumidores. La desinfección es el único paso de la cadena de producción para la inactivación y/o eliminación de bacterias patógenas, así como la reducción de la microflora natural asociada. Tradicionalmente, la industria de productos MPF ha utilizado hipoclorito sódico (NaClO) como desinfectante. Sin embargo, existen diversos problemas asociados a su uso, como su dependencia del pH, la generación de subproductos potencialmente cancerígenos, así como su toxicidad, que puede afectar a la seguridad de los trabajadores y que no siempre es suficientemente eficaz. Además, el NaClO, incluso a baja concentración, puede causar olor y sabor extraño en determinados productos. Por ello, el NaClO está siendo cuestionado, incluso prohibido en determinados países de la Unión Europea como Alemania, Dinamarca, Bélgica y Holanda y previsiblemente se restringirá más su uso, lo que exige disponer de alternativas de desinfección para preservar la seguridad alimentaria a los consumidores. Dentro de las alternativas consideradas como prometedoras se incluyen las soluciones antimicrobianas (agua electrolizada, dióxido de cloro o clorito sódico acidificado), la radiación UV-C y el envasado en atmósferas modificadas activas, enriquecidas en oxígeno, óxido de nitroso o gases nobles. Estas alternativas podrían aplicarse solas o como una combinación de varias técnicas.

Los trabajos de investigación de la presente Tesis Doctoral se centran en el estudio de las diferentes alternativas al NaClO sobre la calidad microbiológica, nutritiva y sensorial de brotes de hortalizas foliáceas MPF y tomate para consumo en fresco. Las

investigaciones se han realizado en el Grupo de Postrecolección y Refrigeración de la Universidad Politécnica de Cartagena y en el Department of Plant Sciences de la Universidad de California, Davis (EEUU), dentro del marco de colaboración que ambos grupos de investigación mantienen.

Los efectos del envasado en atmósferas modificadas (AM) activas, enriquecidas en O_2, He, N_2 y N_2O sobre la calidad de brotes de Red Chard MPF se han comparado en el Capítulo I con los de un tratamiento convencional de desinfección con NaClO y envasado en AM pasiva. El envasado en atmósfera superoxigenada inhibió el crecimiento microbiano durante la conservación refrigerada. El contenido total de vitamina C disminuyó en un 67% para las muestras control (AM pasiva) mientras que en las AM activas la disminución fue menos acusada (50%). La AM activa enriquecida en He mantuvo los niveles de clorofila iniciales hasta el final de la vida útil del producto. Como principal conclusión, las AM activas enriquecidas en He y O_2 resultaron ser herramientas útiles para el mantenimiento de la calidad de Red Chard MPF.

En el Capítulo II se estudia el agua electrolizada (AE) como un higienizante emergente para la industria de la IV Gama. Se detallan los efectos del AE neutra y ácida sobre la calidad total de Mizuna MPF incluyendo cambios fisiológicos, microbiológicos, nutricionales y sensoriales durante la vida útil del producto, comparado con una desinfección convencional con NaClO. Ambos tratamientos desinfectantes mostraron un efecto inhibitorio sobre la microflora natural del producto y los principales atributos de calidad se conservaron durante la vida comercial. Se registró un ligero incremento del contenido inicial de polifenoles totales en las muestras tratadas con AE ácida. Sin embargo, se observó un descenso del 35% de la actividad antioxidante total inicial tras al finalizar su vida útil. Ninguno de los tratamientos afectó a la estructura superficial de los tejidos según los estudios realizados de microscopía electrónica de barrido. Como conclusión más relevante, el AE resultó ser tan efectiva como el NaClO y la calidad global de la Mizuna MPF se mantuvo durante 11 días a 5ºC.

El uso de clorito sódico acidificado (CSA) como desinfectante alternativo al NaClO sobre la microflora natural, inactivación de *Escherichia coli* y la calidad sensorial de Tatsoi MPF se estudia en el Capítulo III. Para optimizar la eficiencia de este desinfectante se utilizó una combinación factorial de los siguientes elementos: dosis de CSA (100, 300 y 500 mg/L), tiempo de contacto (60, 90 y 120 s) y temperatura de almacenamiento (5 y 10ºC). El CSA resultó ser tan efectivo como el NaClO para la inactivación de la microflora natural y de *E. coli* inoculada artificialmente en brotes de Tatsoi. El aumento del tiempo de contacto no supuso un incremento de la actividad antimicrobiana. Los atributos de calidad se mantuvieron durante 11 días a 5ºC, pero para la conservación a 10ºC, solo sucedió durante los 5 primeros días.

En el Capítulo IV se evalúa el riesgo de contaminaciones cruzadas por *E. coli* O157:H7 y *S. enterica* ser. Typhimurium durante la etapa de lavado y desinfección de Red Chard y la acción preventiva del dióxido de cloro (ClO_2) y NaClO como higienizantes. Para ello, se inoculó artificialmente el material vegetal a un nivel relativamente bajo (log 4), se mezcló con material no inoculado y se procesó en una única unidad. Las hojas de Red Chard representaron únicamente el 3-5% del material inoculado total. Los conteos de bacterias antes, durante y después del procesado en hojas y agua de lavado fueron relativamente bajos, por debajo del índice detección cuantitativo. Para la detección se usaron técnicas convencionales así como técnicas de detección rápida basados en PCR en tiempo real. Ninguno de los higienizantes utilizados llegó a prevenir la transferencia de *Salmonella* al agua de lavado y al material vegetal no inoculado. El ClO_2 disminuyó el riesgo de contaminación cruzada por *E. coli* O157:H7, pero no fue capaz de evitarla totalmente. La transferencia de *Salmonella* al agua de proveniente del centrifugado resultó relativamente alta convirtiéndola en un importante punto de control crítico a tener en cuenta por la industria de los productos MPF.

La mayoría de contaminaciones por patógenos alimentarios de las hortalizas de hoja ocurre durante su producción. Entre las principales causas cabe destacar el uso de enmiendas orgánicas contaminadas, contaminaciones cruzadas por manipulación humana en las operaciones precosecha, empleo de agua de riego contaminada y presencia de material fecal por la intrusión de animales en el campo o falta de higiene del personal que trabaja en el campo. Además, los estudios científicos disponibles sobre el comportamiento de patógenos entéricos en los tejidos vegetales, así como durante su procesado, son escasos y no muy claros. El principal objetivo del Capítulo V la evaluación del comportamiento de *E. coli* y *E. coli* O157:H7 sobre hojas de Mizuna, Tatsoi y Red Chard desde la producción hasta su procesado y distribución en un sistema modelo. La habilidad de este patógeno entérico para sobrevivir sobre tejidos vegetales durante las operaciones precosecha hasta la distribución comercial se ha demostrado. Este estudio ha enriquecido la escasa información existente sobre el comportamiento de *E. coli* en brotes de hortalizas foliáceas además de proporcionar datos científicos útiles para el desarrollo de una adeacuada evaluación de riesgos durante la producción y procesado de estas hortalizas.

El objetivo del Capítulo VI fue evaluar el efecto combinado de la radiación UV-C y el envasado en AM activas enriquecidas en O_2, comparado con un envasado convencional en AM pasiva en la calidad total de Tatsoi MPF. La radiación UV-C sola y combinada con el envasado en AM superoxigenada fueron los tratamientos que mejor controlaron el crecimiento de la microflora natural durante la vida útil del producto a 5ºC. Dichos tratamientos no afectaron al contenido total de clorofila, polifenoles y capacidad antioxidante total durante la conservación refrigerada. La radiación UV-C y el envasado en AM enriquecida en O_2 son tratamientos no térmicos de conservación y podrían utilizarse con este propósito en la industria para preservar la calidad total de tasoi MPF, minimizando el consumo de agua.

En el Capítulo VII se evaluó la eficacia del ClO_2 en sistemas industriales para el lavado y desinfección de tomate para consumo en fresco en dos factorías de California y Florida (EEUU). Los sistemas de lavado constaban de un tanque de recepción y un tanque de lavado, provistos de un sistema de recirculación de agua. Se evaluaron regularmente los niveles microbiológicos de los tomates antes y después del lavado, y del agua en cada tanque durante una jornada laboral. Del mismo modo, se evaluaron los parámetros fisicoquímicos temperatura, pH, turbidez, ClO_2 residual, conductividad y potencial de oxidación redox (ORP) del agua de procesado de cada tanque. El sistema fue capaz de eliminar 5 log CFU/tomate de *Pseudomonas flourescens* inoculada artificialmente. Sin embargo, se registraron niveles de aerobios mesófilos totales alrededor de 4 log CFU/fruto después del procesado de tomates microbiológicamente esterilizados con $AgNO_3$. La turbidez y la conductividad del agua de lavado aumentaron con el tiempo, mientras que el pH y el potencial de oxido-reducción se mantuvieron constantes. Se observó una correlación positiva entre el potencial redox y la temperatura, y una correlación negativa entre el potencial redox y la turbidez, la carga de aerobios mesófilos y coliformes totales. Este estudio a nivel industrial ha revelado valiosa información sobre el manejo y las limitaciones de sistemas operados con ClO_2 en industrias de manipulación de tomate fresco.

El diseño de los experimentos recogidos en el Capítulo VIII deriva de los resultados obtenidos en el Capítulo VII, en el que se ha demostrado que la calidad del agua tiene un fuerte impacto sobre la efectividad del ClO_2. El objetivo de esta nueva experiencia fue evaluar la estabilidad del ClO_2 en el agua de lavado de tomate fresco y el efecto antimicrobiano para inactivar diferentes serotipos de *Salmonella enterica*. Se utilizó una combinación factorial de los siguientes elementos: turbidez (0, 22, 43 y 160 FAU), temperatura del agua (10, 25 y 40ºC) y dosis de ClO_2 (1, 3 y 5 mg/L) para evaluar el estado oxidativo del sistema (potencial redox) durante el tiempo y la capacidad para inactivar *Salmonella*. Entre los resultados obtenidos se observó una correlación inversa entre la turbidez del agua y la temperatura. El incremento de la turbidez redujo el potencial redox y aumentó el tiempo de contacto para la inactivación de *S. enterica*. Los resultados obtenidos demuestran el importante efecto de la temperatura y la calidad del agua para el mantenimiento del potencial redox en el tiempo y la inactivación de *Salmonella*.

En la presente Tesis Doctoral se han evaluado diferentes alternativas al NaClO en hortalizas foliáceas y tomate. Se ha demostrado que el ClO_2, el AE y el CSA aplicados en la etapa de lavado reduce la microflora natural del producto así como la de los patógenos inoculados, de manera tan eficaz como el NaClO, preservando los principales atributos de calidad de los productos tratados. Sin embargo, el lavado-desinfección con agentes químicos no garantiza la eliminación total de patógenos alimentarios, pero sí contribuye a su inactivación y disminución del riesgo de contaminaciones cruzadas. Al evaluar la eficacia de un desinfectante a nivel industrial, su efectividad está limitada por las condiciones industriales específicas, las cuales pueden variar de una factoría a otra. En sistemas de lavado industriales de ClO_2

provistos de recirculación de agua, la calidad del agua de lavado tiene un fuerte impacto sobre la efectividad del desinfectante. El lavado-desinfección de los productos constituye solo una parte dentro del sistema de limpieza y desinfección y no debe considerase aislada. Por tanto, es crucial una visión integrada que englobe Buenas Prácticas Agrícolas, Buenas Prácticas de Fabricación y un efectivo sistema de APPCC para ofrecer a los consumidores productos frescos y MPF microbiológicamente seguros y de alta calidad.

ABSTRACT

Nowadays, the beneficial effect of the consumption of fruit and vegetables to human health is well known, in part thanks to the recent scientific and technological advances developed during the last decades. Several epidemiological studies have demonstrated the association of the fruit and vegetables consumption, with powerful antioxidant properties, and the prevention of some kind of cancers and cardiovascular diseases. Moreover, the consumption has been promoted by researchers, nutritionists, and even at a governmental level, that recommends at least 5 servings of fruit and vegetables a day.

The current lifestyle with little time to prepare a convenient meal and a balanced diet has created a new kind of consumer who demands fresh, healthy, and ready-to-eat products. Fresh-cut vegetables (FC) are products that fit within the new consumer trends. These products are elaborated free from additives, packaged under modified atmosphere packaging (MAP), distributed at chilling temperatures and ready to eat. The main advantage of FC plant foods is that they have almost the same properties than the whole intact product, with a much reduced elaboration time and with a uniform and consistent quality.

The association of foodborne outbreaks linked to the consumption of fresh and FC vegetables has dramatically increased during the last years. Thus, the concern about these issues by consumers has also increased. Disinfection is the only step that contributes to effectively reducing microbial load across the supply chain. Sodium hypochlorite (NaClO) has been the most common sanitizing agent in the FC industry. However, some problems have been identified related to its use, such as potentially hazardous disinfection-by-products formation, its strong pH dependence, and the potential for gas emission that may affect worker safety and poor efficiency in several commodities. For these reasons, some European countries such as Germany, Denmark, Belgium, and Holland have forbidden its use in the FC industry and this may lead to new regulatory restrictions in the near future. Due to the combination of issues previously mentioned, it is imperative to investigate new alternatives which could replace NaClO in the FC industry. Antimicrobial solutions (electrolyzed water, chlorine dioxide, and acidified sodium chlorite), UV-C radiation; and packaging under active MAP enriched with oxygen, nitrous oxide, or noble gases have been proposed as alternatives to replace NaClO.

The research of this current Thesis focuses on the evaluation of alternatives to chlorine in the fresh-cut industry, while keeping nutritional and sensory quality of FC baby leafy greens and fresh tomatoes. Investigations have been performed within the cooperation agreement that the Postharvest and Refrigeration Group of the Technical University of Cartagena and the Department of Plant Sciences, University of California, Davis (USD, USA) is currently maintained.

The antimicrobial effects and quality changes of O_2, He, N_2, and N_2O-enriched active MAPs (initially 100 kPa) compared to a chlorinated -100 mg/L NaClO- passive MAP (control) in fresh-cut Red Chard baby leaves up to 8 days at 5ºC were studied in Chapter I. High O_2 MAP inhibited natural microflora growth during the chilling storage. Regarding control treatment, no differences for He and N_2O-enriched MAPs on microbial growth were found, although control samples were previously disinfected. In control samples, vitamin C content decreased up to 67% after the shelf life, while samples subjected to non conventional MAPs decreased by 50%. He-enriched MAP preserved the total chlorophylls content throughout shelf life. It is concluded that He and O_2-enriched MAPs are useful tools in the preservation of fresh-cut Red Chard quality and might serve as an alternative to chlorine in the disinfection step.

Chapter II evaluates the use of electrolyzed water (EW) as an emergent sanitizer for the FC industry. The effects of neutral and acidic electrolyzed water (NEW and AEW) during the washing-disinfection step on quality attributes changes during the shelf life of fresh-cut Mizuna baby leaves were studied. Physiological, nutritional, enzymatic, sensory, and microbial changes throughout 11 days at 5º C compared to a conventional industrial treatment with NaClO were monitored. Both NEW and AEW showed an inhibitory effect on natural microflora growth and retained the main quality attributes throughout the shelf life. Initial total phenolics content ranged slightly increased throughout shelf life for the samples treated with AEW. On the contrary, after shelf life, the total antioxidant activity recorded on the processing day decreased around 35%. Scanning electron microscopy of the leaves showed that neither NEW nor AEW affected their surface structure. In conclusion, EW provides an alternative sanitizing technique to NaClO for maintaining the quality of fresh-cut Mizuna baby leaves up to 11 days at 5º C.

The decontamination efficacy and quality attributes effects of acidified sodium chlorite (ASC) on fresh-cut Tatsoi baby leaves have been investigated in Chapter III. A factorial design of the following factors: ASC dose (100, 300 and 500 mg/L), time contact (60, 90 and 120 s) and storage temperature (5 and 10ºC) was used to optimize the sanitizer. ASC 100-500 mg/L showed an initial antimicrobial efficacy on reduction of epiphytic microflora and *Escherichia coli* as effective as NaClO on inoculated leaves. No significant differences on antimicrobial effect of ASC within 60, 90 and 120 seconds of contact time were found. Sensory quality attributes of fresh-cut leaves were well kept at 5ºC up to 11 days, but at the abusive temperature of 10ºC only 5 days was assured. As a main conclusion, ASC provides an alternative sanitizing technique to NaClO for maintaining the quality and safety of fresh-cut Tatsoi baby leaves up to 11 days at 5º C.

Chapter IV evaluates process handling cross-contamination potential and pathogen removal of initially low numbers of attached cells of *Salmonella* and *E. coli* O157:H7 during the washing-disinfection, rinsing, and de-watering steps of fresh-cut Red Chard as affected by ClO_2 and NaClO. Red Chard leaves were artificially

inoculated at a low inoculums level (log 4), mixed with non-inoculated leaves and processed as a unit. Inoculated material represented the 3-5% of the total within the same lot. Detection and recovery was performed by using conventional and PCR-based techniques. Selected levels of ClO_2 and NaClO used in this experiment were unable to fully disinfect the applied pathogen surrogates from inoculated leaves regardless of the washing type. While ClO_2 substantially prevented *E. coli* O157:H7 cross contamination, of the isolate used in this study, it was not effective for the *Salmonella* isolate. Large populations of *Salmonella* were recovered from centrifugation discharge effluent water whereas no colonies were detected from water in contact with inoculated leaves in earlier washing unit operations. This represents a potential risk of cross-contamination transference to product and equipment at the step immediately prior to packaging.

Human cross-contamination during preharvest practices and postharvest handling, fecal contamination by animals, use of untreated manure and application of contaminated irrigation water have been identified as potential sources of contamination of vegetables. Moreover, scientific based data about the survival of enteric pathogens in the phyllosphere of leafy vegetables and after processing and retail distribution is scarce and relatively unclear. The objective of the Chapter V was to assess the fate of *E. coli* and *E. coli* O157:H7 in Mizuna, Tatsoi and Red Chard from preharvest to postharvest processing, packaging and storage conditions in a model system. Genotyping of generic *E. coli* strains to evaluate their distribution in the phyllosphere from production throughout processing is also reported. The ability of survival during production and after disinfection as well as storage of low levels of *E. coli* in the assayed mini greens has been demonstrated. However, field-based trials under realistic conditions to understand the fate of the pathogen should be further studied. In summary, this research provides useful data to develop an adequate science-base risk assessment during the production and minimal processing of these crops.

The objective of Chapter VI was to evaluate the effects of a moderate UV-C pretreatment, a superatmospheric O_2-enriched modified atmosphere packaging (MAP) and its combination compared to a conventional passive MAP on microbial, nutritional and sensory changes of fresh-cut Tatsoi baby leaves throughout 11 days at 5° C were investigated. The UV-C and the combined UV-C + O_2-enriched MAP were the treatments which best controlled the epiphytic microbial growth. Treatments assayed did not affect the total chlorophyll, phenolic content, and the total antioxidant capacity, which were kept throughout storage. According to sensory quality, the shelf life was 9 to 10 days at 5° C for all treatments. These innovative sustainable combined treatments could be useful to the industry for keeping overall quality of fresh-cut Tatsoi baby leaves, and probably other leafy vegetables, while minimizing water consumption.

Chapter VII aims to obtain efficacy data for whole system chlorine dioxide (ClO_2) treatment as a baseline for operating standards in dump tank and flume systems within tomato packing operations. On-site assessments were recorded in three packinghouse facilities (two in Florida (FL), one in California (CA)). Incoming

tomatoes were collected every 30 minutes at the end spray-wash bed for microbiological analysis of mesophiles, coliforms, and *Escherichia coli*. Water was collected from dump and flume systems, and their respective re-circulation tanks every 30 minutes for equivalent microbiological content. Other parameters monitored were water temperature, pH, ClO_2 residual, turbidity, conductivity, and oxidation reduction potential. The system was able to remove around 5 log CFU/fruit of inoculated *Pseudomonas fluorescens*. However, surface sterilized tomatoes acquired approximately 4 log CFU/fruit of total aerobic mesophilic but not coliforms after washing. Turbidity and conductivity increased during this time due to the associated organic matter from the incoming tomatoes and water temperature; while pH and oxidation-reduction potential (ORP) remained mostly constant during this time. ORP levels were significantly higher in flume than in dump tanks. A significant and positive correlation was found between ORP and temperature, while a significant and negative correlation occurred between ORP and turbidity as well as total mesophiles and coliforms. Facilities were not able to maintain a temperature differential above 5.5° C between water and fruit pulp during the full daily operation, as recommended by the tomato food safety guidelines. As a main conclusion, this study provides in-plant data that ClO_2 is an effective sanitizer for flume and spray-wash systems, but current operational limitations greatly restrict performance in dump tank management.

The experimental design of the research work presented in Chapter VIII results from the findings obtained in the Chapter VII, where it has been demonstrated that water quality affects the ClO_2 effectiveness. Water disinfection is a critical control point to minimize transmission of bacterial pathogens from water sources to fresh produce. This work aims to establish a correlative capacity of the Oxidation Reduction Potential (ORP) and ClO_2 dose, under different conditions of water turbidity and temperature, and their potential to inactivate *Salmonella enterica* on tomato processing water. ORP was monitored under 1, 3 and 5 mg/L ClO_2, water temperature (10, 25 and 40ºC) and turbidity (0, 40 and 160 FAU). The main results show inverse correlations between water turbidity, temperature and ORP. An increase in turbidity significantly reduces the final ORP and increases the contact time required for inactivation of *S. enterica* at any assayed temperature. An increase in temperature and ClO_2 concentration reduces the contact time and achieves a 6-log reduction of *S. enterica* within a 2 minute time frame. However, water composition strongly affects the ORP values, which can limit total inactivation of *S. enterica*. Generated data demonstrated the effect of water quality and temperature to maintain an effective ORP towards inactivation of *S. enterica*, which could impact the current definition of adequate water quality and safety standards.

The proposed alternatives to NaClO in baby leafy vegetables and tomatoes have been evaluated in the current Doctoral Thesis. The use of ClO_2, ASC, EW applied during the washing-disinfection step, reduces the natural microflora as the utilized foodborne pathogens as effective as chlorine, while keeping overall quality of the products. However, the application of chemical sanitizers during the washing-disinfection step does not guarantee the total inactivation of pathogens and avoidance of

the cross-contamination. When evaluating the efficacy of a sanitizing agent at industrial level, results may vary among factories and depend on the specifics conditions. The application of a sanitizer or a combination of disinfection treatments does not mean a sanitation program. It implies much more effort from a well designed integrated production, handling, and processing to proper distribution chains, keeping appropriate chilling storage temperatures and optimal MAP conditions throughout the entire commercial life. In addition, Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP), and Hazard Analysis and Critical Control Points (HACCP) programs should be implemented and accomplished to minimize the risk of contamination and to assure safety and overall quality to consumers.

LIST OF ABBREVIATIONS

µg: micrograms

A: Absorbance

AA: Ascorbic acid

AEAC: Acid equivalent antioxidant capacity

AEW: Acidic electrolyzed water

AFHORLA: Asociación Española de Frutas y Hortalizas lavadas, listas para su empleo

ANOVA: Analysis of variance

AOAC: Association of Official Analytical Chemists

APPCC: Análisis de Peligros y Puntos de Control Críticos

ASC: Acidified sodium chlorite

ATCC: American Type Culture Collection

atm: Atmosphere

ATP: Adenosine triphosphate

ATSDR: Agency for Toxic Substances and Disease Registry

BOPP: Bi-oriented polypropylene

BPW: Buffered peptone water

BSL: Biosafety level

C^*: Chroma

C: Celsius

CA: California

CDC: Center for Disease Control and Prevention

CDPH: California Department of Public health

CFR: Code of Federal Regulations

CFU: Colony-forming unit

ChAE: Chlorogenic acid equivalent

Chl *a*: Chlorophyll a

Chl *b*: Chlrophyll b

cm: Centimeters

COD: Chemical Oxygen Demand

Ct: Cycle threshold

cv.: Cultivar

d: Days

DHA: Dehydroascorbic acid

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EPA: Environmental Protection Agency

EU: European Union

EW: Electrolyzed Water

FAO: Food and Agriculture Organization of the United Nations

FAU: Formazin attenuation units

FC: Fresh-cut

FDA: Food and Drug Administration

FDACS: Florida Department of Agriculture and Consumer Services

FID: Flame ionization detector

Fig.: Figure

FL: Florida

FRAP: Fluorescence Recovery After Photobleaching

fw: fresh weight

g: grams

g: Gravitational acceleration

GAP: Good Agricultural Practices

GC: Gas Chromatography

GMP: Good Manufacturing Practices

GPR: Grupo de Postrecolección y Refrigeración

GRAS: Generally Recognized As Safe

h: hours

Ha: Hectare

HACCP*:* Hazard Analysis Critical Control Point

Hz: Hertz

IARC: International Agency for Research on Cancer

IPL: Intense Pulse Lights

J: Juole

kg: kilogram

kJ: kilojoules

km: kilometres

kPa: kilopascal

L*: Lightness

L: Liter

LAB: Lactic acid bacteria

LOD: Limit of detection

log: Logarithm

LSD: Least significant difference

M: Molar

MAP: Modified atmosphere packaging

MFP: Minimally fresh processed

mg: milligram

min: minute

mM: millimolar

mm: millimeter

MPa: megapascal

MPN: Most probable number

MRS: de Man, Rogosa and Sharpe

mS: millisiemens

ms: millisecond

mV: millivolt

N: Normal

NATTWG: North American Tomato Trade Work Group

NEW: Neutral Electrolyzed Water

nm: nanometer

ns: nanosecond

OD: Optical density

ORP: Oxide reduction potential

PA: Peroxiacetic acid

PAL: Phenylalanine ammonia lyase

PCA: Plate count agar

PCR: Polymerase chain reaction

PDA: Potato dextrose agar

pH: potential hydrogen

PP: Polypropylene

ppm: Parts per million

PPO: Polyphenol oxidase

qRT-PCR: Quantitative real time PCR

R: Pearson correlation coefficient

RD: Real decreto

REP-PCR: Repetitive extragenic palindromic PCR

RH: Relative humidity

Rif: Rifampicin

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RR: Respiration rate

s: seconds

SAS: Statistical Analysis System

SD: Standard deviation

ser.: Serotype

SOD: Superoxide dismutase

SOP: Standard operation procedure

spp.: species

SSC: Soluble solid content

sv.: serovar

TA: Total acidity

TAM: Total aerobic mesophilic

TCD: Thermal conductivity detector

THM: Tri-halo-methane

TSA: Tryptic soy agar

TSB: Tryptic soy broth

UCDavis: University of California, Davis

UK: United Kingdom

UPCT: Universidad Politécnica de Cartagena

US: United States

USA: United States of America

USDA: United States Department of Agriculture

UV: Ultraviolet

V: Volt

v/v: volume/volume

v/w: volume/weight

VRBD: Violet red bile dextrose

w/w: weight/weight

XLD: Xylose lysine deoxycholate

INTRODUCTION

1. AGRONOMIC AND ECONOMIC ASPECTS OF BABY LEAFY VEGETABLES IN SPAIN

1.1. Baby leafy vegetables

Baby leafy vegetables or mini-greens have a cosmopolitan distribution around the world. In particular there are significant concentrations in the Mediterranean areas including the Murcia Region, as well as in some regions of the South-West and Central Asia. Baby leaves can be industrially used to be included in mixed lettuce based salads. Along with their nutritional value, they add a piquant distinctive taste. At mid maturity (8-15 cm leaf size) they can be also used in stir fry dishes (Martin, 2008).

The selected baby leafy vegetables included in the experiments accomplished in this current Thesis are Tatsoi, Mizuna, and Red Chard. The development and production of these crops is still in early stages in Spain and there are no official available facts. The main producer in the Murcia Region is "Grupo G's España" (Torrepacheco, Murcia, Spain). According to the available data of this company, the processing harvested Tatsoi and Red Chard in 2010 was 10 and 20 Ha respectively and the production was around 35,000 and 105,000 kg per year.

1.1.1. Tatsoi

Order: *Brassicales,* Family: *Brassicaceae,* Genus: *Brassica,* Name: *Brassica rapa* cv. rosularis.

Figure 1.1. Tatsoi baby leaves.

Tatsoi, also called spinach mustard, spoon mustard, or rosette bok choy, is an Asian variety of *Brassica rapa* grown for greens. It is a cold tolerant leafy vegetable (-10ºC) and it grows in a rosette form (15-25 cm). While it is tolerant of cold or even light snow, it deteriorates in prolonged wet weather. The leaves are spoon-shaped, dark green, and waxy with a short green petiole (2-9 cm) on the leaf stalk. It takes 45-50 days to harvest. The baby size (7-10 cm) has become a foundation ingredient for mixed

salads. It has a soft creamy texture and has a subtle yet distinctive flavor. At maturation, the leaf stalk and blade can reach 25 cm long. Nutritionally, Tatsoi is a valuable source of phosphorous, calcium iron, potassium, and vitamins A and C (Martin, 2008).

1.1.2. Mizuna

Order: *Brassicales,* Family: *Brassicaceae,* Genus: *Brassica,* Name: *Brassica rapa* cv. japonica.

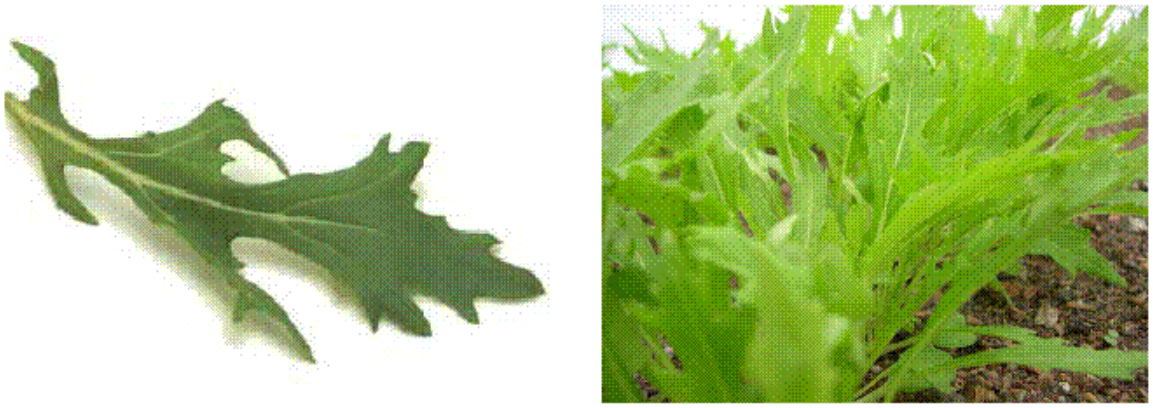

Figure 1.2. Mizuna baby leaves.

Mizuna, also called Xiu Cai, Kyona, Japanese Mustard, Potherb Mustard, Japanes Greens, California Peppergrass, Spider Mustard, etc., is a Japanese name used primarily for cultivated varieties of *Brassica rapa* cv. japonica. Mizuna is characterized for its easy cultivation (in different soil types) and its reproductive capacity. Spring is the best time for cultivation. Mizuna has also a good resistance against heat and cold (no lower than 2°C). The harvesting of Mizuna can begin 20-60 days after planting, according to the period of growth, climate, and environmental conditions. These species have the capacity to grow again after the first harvest; the second harvest occurs in 10-30 days. The number of crops varies greatly with the species, but usually only 2 or 3 harvests are carried out depending on the season (Pimpini and Enzo, 1996). Mizuna is low in calories, high in folic acid (vitamin B9), high in vitamin A and carotenoids, high in vitamin C, and it contains glucosinolates which are antioxidants which help to prevent certain cancers. Dark green leaves offer the most nutrition and provide beta-carotene, vitamins, minerals, and plus they are a source of fiber (Pimpini and Enzo, 1996, Martin, 2008).

1.1.3. Red Chard

Order: *Caryophyllales,* Family: *Amaranthaceae,* Genus: *Beta,* Name: *Beta vulgaris* cv. cycla

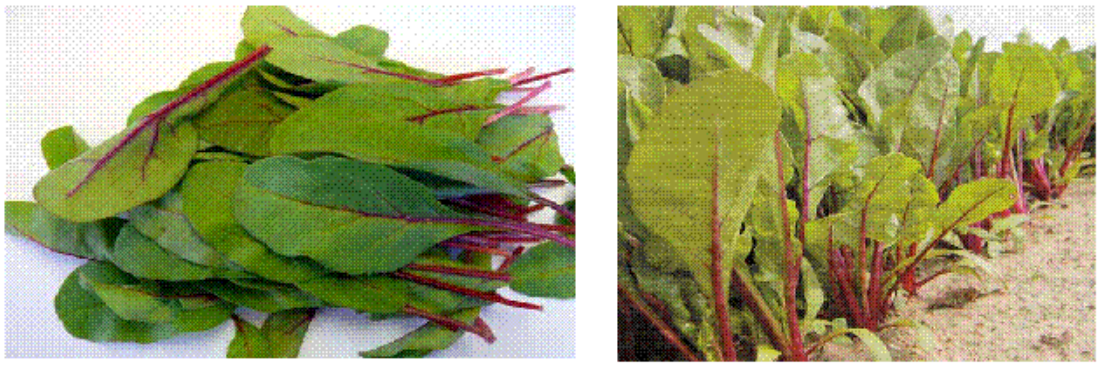

Figure 1.3. Red Chard baby leaves.

Red Chard is a popular red-stemmed chard. Chard can be harvested while the leaves are young and tender and is extremely perishable. The harvesting of Red Chard commonly begins 50-60 days after planting, according to the period of growth, climate and environmental conditions. Fresh young chard can be used raw in salads. Mature chard leaves and stalks are typically cooked. The flavor is mild yet earthy and sweet with slightly bitter undertones. Several studies have reported the nutritional contents of these fresh leaves. Pyo *et al.* (2004) reported that the antioxidant activity and phenolic compounds of Swiss Chard extracts are a good dietary source of phenolic antioxidants. In addition, Red Chard is rich in vitamin C (Gil *et al.*, 1998).

2. AGRONOMIC AND ECONOMIC ASPECTS OF TOMATO IN THE UNITED STATES

The tomato is native to South America. After the Spanish colonization of America, the Spaniards distributed the tomato throughout their colonies in the Caribbean and Philippines. Tomatoes were also brought to Spain and then spread to the rest of Europe.

Order: *Solanales,* Family: *Solanaceae,* Genus: *Solanum,* Name: *Solanum lycopersicum* (synonimus *Lycopersicum esculentum* and *L. lycopersicum*).

Figure 2.1. Tomato production.

The first producers of tomatoes in the world is China (33,811,722 tons) followed by United States (12,300,900 tons) and Turkey (10,985,400 tons), while Spain ranked 9th with 3,847,800 tons (FAOSTAT, 2008). In California, tomatoes are grown in the San Joaquín and Sacramento Valleys, mainly centered in Fresno, Yolo, San Joaquín, Kings, and Colusa Counties. Significant production also occurs in Merced, Stanislaus, Solano, and Sutter Counties. Fields are planted from late January through early June for continuous harvest from late June into October. California accounts for over 90% of U.S. production and approximately 35% of world production (Hartz *et al.*, 2008).

Table 2.1. Processing harvested tomato (Ha), yield per hectare (T) and production in the United States and California during 2008, 2009 and 2010.

	Harvested (Ha)		
	2008	*2009*	*2010*
United States	119,964	132,628	115,716
California	112,479	124,616	108,433
	Yield per hectare (tons)		
	2008	*2009*	*2010*
United States	16.79	17.24	18.13
California	17.14	17.49	18.57
	Production (tons)		
	2008	*2009*	*2010*
United States	12,305,820	13,970,560	12,819,110
California	11,822,000	13,314,000	12,300,000

Source: USDA National Agricultural Statistics Service, http://www.nass.usda.gov.

Tomatoes are a warm-season crop that is sensitive to frost at any growth stage. The optimal soil temperature for seed germination is 20°C or above; germination below 16°C is extremely slow. Daily maximum air temperatures between 25 to 35°C are ideal for vegetative growth, fruit set, and development. With adequate soil moisture, tomato plants can tolerate temperatures well in excess (38°C); although fruit set can be

adversely affected. Fruit development and quality are severely reduced when day and night temperatures fall below 20 and 10ºC, respectively (Hartz *et al.*, 2008).

A wide variety of soil textures are used for tomato production. Sandy soils are preferred for early plantings, because planting can be done in sandy soils more easily during wet weather. Sand also warms more rapidly in the spring, promoting early growth. However, loam and clay loam soils are generally more productive than sand. Clay soil may be used if it is well drained and irrigated with care (Hartz *et al.*, 2008).

Surface drip irrigation is used primarily in pole production, while in bush production, subsurface drip irrigation is commonly used (Le Strange *et al.*, 2000). Fertilizer application rates vary widely among California tomato growers. Typical seasonal application rates are 140-280 kg/Ha of nitrogen (N) for bush-grown tomatoes and 168-392 kg/Ha for pole-grown tomatoes. Phosphorous (P) application rates of 67-134 kg/Ha of P_2O_5 are adequate for the majority of tomato fields. Typical application of potassium (K) ranges from 0-134 kg/Ha of K_2O depending on the soil (Le Strange *et al.*, 2000).

Tomato is a natural source of lycopene, one of the most powerful natural antioxidants. Tomato consumption has been associated with decreased risk of some kind of cancer and cardiovascular diseases (Giovannucci, 1999).

3. MINIMAL PROCESSING OF LEAFY VEGETABLES

3.1. Introduction

Fresh-cut (FC) or minimally processed (MP) vegetables are ready-to-eat products, elaborated free from additives by using light combined methods such as: washing, cutting, disinfecting, and packaged at chilling temperatures under polymeric films able to generate optimum modified atmosphere packaging (MAP) conditions (Artés and Allende, 2005). The main characteristic is that FC produces are alive even after processing, keeping this living fresh state throughout the shelf-life. FC vegetables usually do not need further processing prior to consumption (Artés and Artés-Hernández, 2000; 2003).

The current lifestyle with scarce time to prepare a convenient meal and to have a balanced diet has created a new kind of consumer who demands fresh, healthy, and ready-to-eat products. FC vegetables fit these consumer expectations and in the last years, the demand of FC products has increased dramatically. The high suitability and nutritional value of FC vegetables present many advantages for consumers and food services (Artés, 2000; Wiley, 1994; Beuchat, 2002; Bruhn, 2002; Artés, 2004):

- Reduce preparation time.
- Provide a uniform and consistent high quality at reasonable price.
- Easy to store and require little storage space.
- Ready to eat.

- Similar characteristics to the original vegetable.
- Low waste wage.

3.2. Current status of the FC produce industry

The FC products sector was developed in the US at the end of the 70s and its original purpose was to supply the fast food establishments. Subsequently, FC products were introduced in Europe around 1980, mainly in France and the United Kingdom (UK). In Spain, the FC products began to be marketed in the 90s (Sánchez-Pineda, 2003).

According to the latest available data from "Asociación Española de Frutas y Hortalizas lavadas, listas para su empleo" AFHORLA (2008), the total production of FC fruit and vegetables in Spain was around 67,000 tons in 2009, representing an increase of 6.4% compared to 2008. An amount of 65,000 tons of the total sales were vegetables and the rest were fruits. The Spanish FC produce industry reached a turnover of approximately 200 M€ in 2009.

About 7.7 million of households bought at least one FC product a year in 2007 according to AFHORLA (2008). The average purchases of this kind of products were about 2.8 kg per person and year. Despite the gradual increasing of consumption, these facts are still far behind compared to other European Union (EU) countries such as the UK or France, where more than 12 and 6 kg per person and year are consumed respectively.

As previously stated, most of the FC products are vegetables and the green salads corresponded to 65% of the total production. The 79% of the total production is distributed to retailers and the remaining 21% to hotels, restaurants, and catering services sector.

3.3. General processing and unit operations

The minimal processing system of vegetables can vary depending on the product. The general flow chart of the process is showed below and based on Artés-Hernández *et al.* (2010). The diagram shows the general unit operations and the maximum recommended temperatures for each processing step in the production line of FC leafy vegetables.

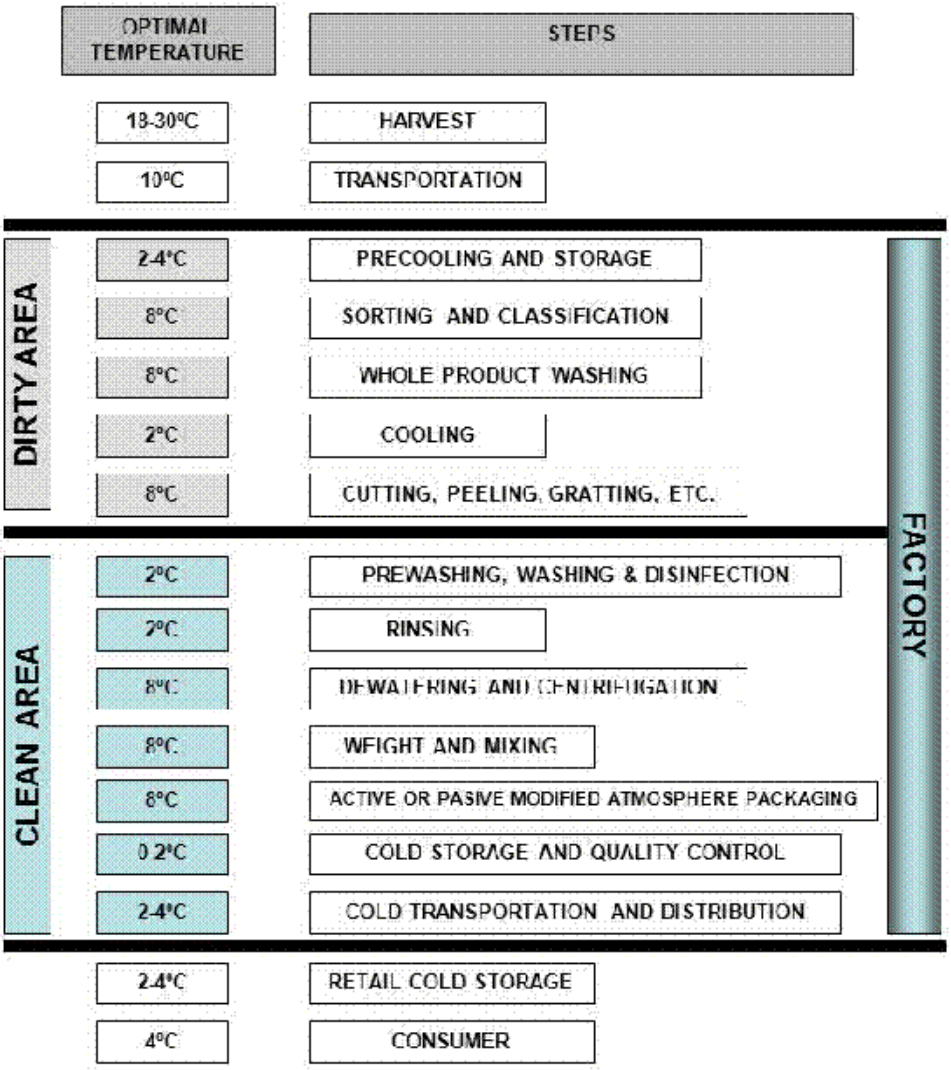

Figure 3.1. Unit operations of minimal processing of leafy vegetables.

3.3.1. Raw material

Many authors had reported the different factors such as irrigation type, climate conditions, soil type, and fertilization, which can affect the physiological behavior and the suitability for fresh processing (Klein and Perry, 1982; King and Bolin, 1989; Brackett, 2000). Among the main factors affecting the quality and shelf-life of vegetables are included (Yildiz, 1994):

- Genetic factors
- Climate conditions (light, temperature, relative humidity, pluviometry, etc.)
- Soil conditions (soil type, pH, humidity, microbiota, mineral composition)
- Agricultural practices (fertilization, pesticides, irrigation type, pollinisation, etc.)
- Harvest (mechanical or manual harvesting, temperature, hygrometry, etc.)

The election of the cultivar is also important because not all varieties of a specific vegetable can be suitable for minimal processing. Some examples are that several research studies have been accomplished to select lettuce cultivars with low sensitivity to browning development after minimal processing (Tomás-Barberán *et al.*, 1997; Castañer *et al.*, 1999), tomato cultivars well adapted to cutting (Aguayo *et al.*, 2001) and suitability of melon cultivars to minimal processing (Silveira *et al.*, 2007).

3.3.2. Transportation

The transportation time from production to processing has to be as short as possible. In addition, transportations that surpass 6 hours after harvesting are strongly recommended to be done at temperatures below 10ºC within the transport medium.

3.3.3. Quality assessment

The first operation upon reception of the raw materials at the factory is quality control; a necessary procedure for a standard product quality. The main criteria are the physical appearance of the raw vegetables, including overall freshness, the absence of insects, physiological and microbial diseases, presence of dirty and necrotic tissues, and compliance with regulations on pesticide residues and nitrate content.

3.3.4. Precooling

The objective of the precooling step is to quickly remove the heat that the plant material brings from the field after harvesting. The low temperature reached in a short time reduces the metabolism of the vegetables. Therefore, the stress generated during the minimal processing operations will be retarded, maintaining the quality of the produce. The main advantages of precooling technique are listed below (Artés, 1987):

- Reducing weight loss and wilting
- Inhibiting microbial growth
- Inhibiting the respiration rate and decay
- Inhibiting ethylene emission and its sensitivity

The precooling system has to be chosen according to different factors such as the type of vegetable, its chilling sensitivity, the type of harvesting containers and economic elements (Artés and Artés-Hernández, 2003). Vacuum cooling is generally well adapted for baby leafy green vegetables, being the most efficient precooling technique.

Figure 3.2. Vacuum cooling system.

3.3.5. Prewashing

Incoming vegetables from the field are commonly cleaned before processing. However, some industries skip this step in certain leafy vegetables. The objective of this step is to eliminate unwanted dirt, pesticide residues, plant debris, insects and foreign matter, and retard the enzymatic discoloration reactions (Allende and Artés, 2005). This unit operation is carried out in the dirty area of the processing line. The use of some sanitizing agent such us sodium hypochlorite (NaClO) in this step is optional and depends on the type of vegetable.

3.3.6. Cutting

This step is one of the most critical points in the minimal processing line. The cutting destroys plant cells and tissues that release nutrients. This process also increases the rate of senescence of tissues and reduces their resistance to microbial spoilage (Artés *et al.*, 2009). These changes are mainly due to the increase of respiration, ethylene production, and mechanical injuries, which result in the release of intracellular oxidizing enzymes and lead various biochemical deteriorations such as browning (Varoquaux and Wiley, 1994; Ahvenainen, 1996). The cutting equipment should be cleaned, disinfected and sharpened at regular intervals every working day. Standard Operation Procedures (SOPs) for cleaning and maintenance of the cutting equipment must be established by the industry.

Figure 3.3. Cutting operations.

3.3.7. Washing and disinfection

Washing and disinfection is a key step that solely contributes to effectively reducing the microbial load across the production chain (Suslow, 1997; Artés *et al.*, 2009). Fresh produce used as raw materials may be a vehicle for transmitting infectious diseases (Leistner and Gould, 2002; Harris *et al.*, 2003; Artés and Allende, 2005). Washing with water physically removes microorganisms from the surfaces of fresh plant produces; however, previous reports have shown few decontamination effects on several whole and fresh-cut commodities (Ruíz-Cruz *et al.*, 2007; Park *et al.*, 2008). In fact, washing with water does not guarantee the inactivation of foodborne pathogens (Artés *et al.*, 2009).

Chlorine, in various forms, has been the most widely used disinfectant in the FC industry. Chlorination of water is one of the primary elements of a properly managed postharvest sanitation program. Water quality management is considered the most important control point in minimizing the transmission of pathogens. Transmission commonly occurs when pathogens from infested plant produce, water, or debris are transferred to non-infested surfaces such as those that are mechanically injured during harvesting, transportation, handling or processing, wounds, or the natural plant surface openings (Artés *et al.*, 2009).

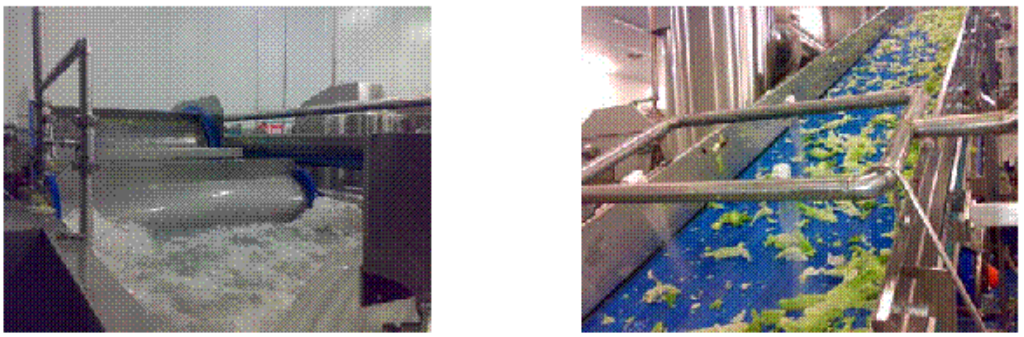

Figure 3.4. Washing-disinfection and rinsed.

NaClO is the most commonly used by the FC industry due to its strong oxidizing properties, antimicrobial activity and low cost. However, some problems have been identified related to its use, including its potentially hazardous disinfection-by-products formation, its strong pH dependence, and the potential for gas emission that may affect worker safety. Recently, Artés *et al.*, (2009) have proposed different sustainable sanitizing alternatives to NaClO for the FC industry. These techniques include alternative antimicrobial solutions, UV-C radiation, ozone, superatmospheric oxygen packaging and innovative gas treatments.

3.3.8. Dewatering and centrifugation

Drying or dewatering of wet surfaces must be carried out carefully to avoid unnecessary damage to the plant tissues, reduce the product moisture content and

remove cell leakage that can promote microbial growth (Simons and Sanguansri, 1997; Soliva-Fortuny and Martín-Belloso, 2003). Dewatering systems include draining systems, gentle removal with cheesecloth, centrifugal spin driers, vibrating racks, rotating conveyors, hydro-sieves, and forced air spinless drying tunnels (Simons and Sanguansri, 1997; Sapers and Miller, 1998; Gorny *et al.*, 2002). Cold air injected over a perforated conveyor belt, which transports the products, has been applied as an alternative to the conventional dewatering systems. However, their main inconvenience is the low efficiency of drying large volumes of product (Artés and Artés-Hernández, 2003).

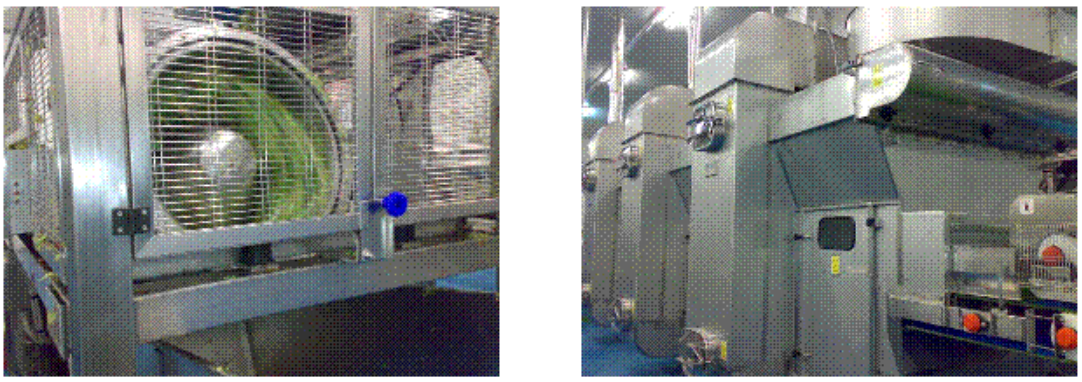

Figure 3.5. Automatic centrifuge and drying tunnel by cold air.

3.3.9. Packaging

Packaging is a key operation in FC vegetables. The most important packaging method for preparing FC vegetables is MAP. In general, MAP is a technique used for prolonging the shelf-life of FC vegetables which involves the reduction of O_2 and elevation of CO_2 concentrations compared to air within the packages. The basic principle in MAP is that a modified atmosphere can be created by using a proper packaging material which must have an appropriate (and often very selective) permeability to gases of physiological interest (O_2, CO_2, C_2H_4, and H_2O). The aim of MAP is to create an optimal gas balance inside the package by the diffusion of these gases generated during respiration under a concentration gradient (Kader, 2002).

There are two types of MAP depending on the kind of generation (Kader, 2002; Artés *et al.*, 2006):

- Passive MAP can be achived if the commodity and film permeability characteristics are properly matched. An appropriate atmosphere can passively evolve within a sealed packaged through consumption of O_2 and production of CO_2 by respiration. The gas permeability of the selected film must allow O_2 to enter the package at a rate offset by the consumption of O_2 by the commodity. Similarly, CO_2 must be vented from the package to offset the production of CO_2 by the commodity.

- Active MAP can be accomplished by creating a slight partial vacuum and replacing the main proportion of the package atmosphere with a desired gas mixture. This mixture can be further adjusted through the use of absorbing substances in the package to scavenge O_2, CO_2, or C_2H_2. Although active MAP implies some additional costs, its main advantage is that it ensures the rapid establishment of the desired atmosphere.

For both cases, a well designed MAP is necessary to maintain the quality during the shelf-life. O_2 concentrations within the package must remain above the extinction point of fermentation. O_2 concentrations that fall below the extinction point can induce anaerobic respiration which can potentially injure the tissues and produce off-flavors in the produce. (Watada *et al.*, 1996).

The main advantages of MAP for the preservation of fruit and vegetables are (Artés, 2000):

- Reduction of respiration rate
- Reduction heat emission from respiration
- Inhibition of ethylene activity and consequently senescence
- Inhibition of sugar, vitamin and organic acid losses
- Total or partial limitation of physiological changes, such as chilling injuries, scalding, browning, etc.
- Inhibition of microbial growth

3.3.10. Quality control

Each factory must implement an effective quality control system to the whole supply chain and the environment to guarantee the safety, suitability and compliance of specifications. In addition, it has to have a procedure of product recall when the specifications are not meet. Instrumentation equipments have to be revised, adjusted and calibrated frequently.

3.3.11. Marketing and retail distribution

Temperature is the most important factor that influences the shelf-life of FC products. Although products are kept at 1-5ºC throughout the distribution chain, it is impossible to guarantee that this temperature will be maintained during transit, distribution and retail display (Artés and Artés-Hernández, 2003). It has already been demonstrated that processed products are often subjected to temperature abuse of about 12ºC in the display cabinets in supermarkets. Inadequate temperature management during marketing and retail distribution coupled with excessive temperature fluctuations during storage can result in the formation of water condensation in the package, and can cause consumer rejection. To prevent abusive temperatures during transport and distribution, Time-Temperature Integrators (TTI) have been used on packages.

However, financial and environmental costs have limited the implantation of this technique (Artés and Artés-Hernández, 2003).

3.4. Physiological and biochemical changes

FC vegetables generally have higher respiration rates than the corresponding intact products. Higher respiration rates indicate a more active metabolism and, usually, a faster deterioration rate (Cantwell and Suslow, 2002) which can also result in more rapid loss of acids, sugars, and other components that determine flavor quality and nutritive value. Physical damage or wounding caused by preparation can increase respiration and ethylene production within minutes and may also increase the rates of other biochemical reactions responsible for changes in color. The importance of temperature is totally implicated in these biochemical processes (Cantwell and Suslow, 2002).

The most important enzyme in FC vegetables is polyphenol oxidase (PPO) which causes browning (Varaquaux and Wiley, 1994; Wiley, 1994). Another important enzyme is lipooxygenase which catalyses peroxidation resulting in the formation aldehyde and ketone derivatives that characterize numerous off-odors.

3.5. Overall quality of FC vegetables

Consumers expect FC products to be fresh and at optimum maturity without defects. These conditions cover the general appearance, sensory quality (texture/firmness, aroma, and taste) and nutritional quality (Watada and Qi, 1999).

FC vegetables are vulnerable to discoloration because of damaged cells and tissues, as well as lack of protective skin. These exposed tissues have the potential of becoming dehydrated and/or discolored. Browning of cut surfaces is a big problem in many FC products; because oxidation of phenols catalyzed by PPO results in the browning complex. The phenols are a product of a reaction catalyzed by phenylalanine ammonia-lyase (PAL), and the activity of PAL is used as an index for potential browning (Mateos *et al.*, 1993). MAP is effective in delaying and/or inhibiting PAL activity (Watada and Qi, 1999).

Cultivars differ in the degree of browning. Thus, selection of a proper cultivar is important to control browning of cut products (Tomás-Barberán *et al.*, 1997).

The general quality condition, which includes: appearance, firmness/texture, and nutritional content is affected by various factors. The most important factor is temperature. When temperatures increase from 0 to 10ºC, the RR increases substantially. With increased RR, deterioration increases at a comparable rate; thus, low temperature is essential for maintaining good quality (Watada *et al.*, 1996). Produce types and cultivars differ in chilling sensitivity and consequently, the optimum storage temperature depends on the product itself and the exposure time.

FC vegetables are highly susceptible to weight loss because internal tissues are exposed if there is a lack of skin and cuticle. However, relative humidity (RH) is generally very high within the package and dehydration is not a big problem. MAP can be beneficial in keeping RH and maintaining quality of the product (Artés, 2000).

3.6. Safety aspects of FC vegetables

A food hazard is any biological, chemical, or physical agent which can cause an illness in the absence of its control. Microbiological safety is the major issue of concern in the FC industry, and risks can be divided into two categories (Hurst, 2002). One category concerns the factors or conditions contaminating fresh produce with indigenous pathogens during cultivation or at harvest. These have been addressed by Hedberg *et al.* (1994) and Tauxe *et al.* (1997). This includes poor agronomic practices, use of contaminated water for crop irrigation or mixing chemical sprays, application of improperly composted manure as fertilizer, and lack of training among field workers on good personal hygiene. Poor sanitary control during harvesting and postharvest handling practices is another mechanism for pathogen contamination to fresh produce.

A second category of microbiological risk is the cutting operation in the minimal processing plant. Internal tissue of fresh produce is normally protected from microbiological invasion by natural barriers such as waxy outer skins or peel. However, cutting operation releases nutrients, which can accelerate microbiological growth, including potential human pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes.*

The Fig. 3.6. shows different mechanisms by which produce can become contaminated with pathogenic microorganisms.

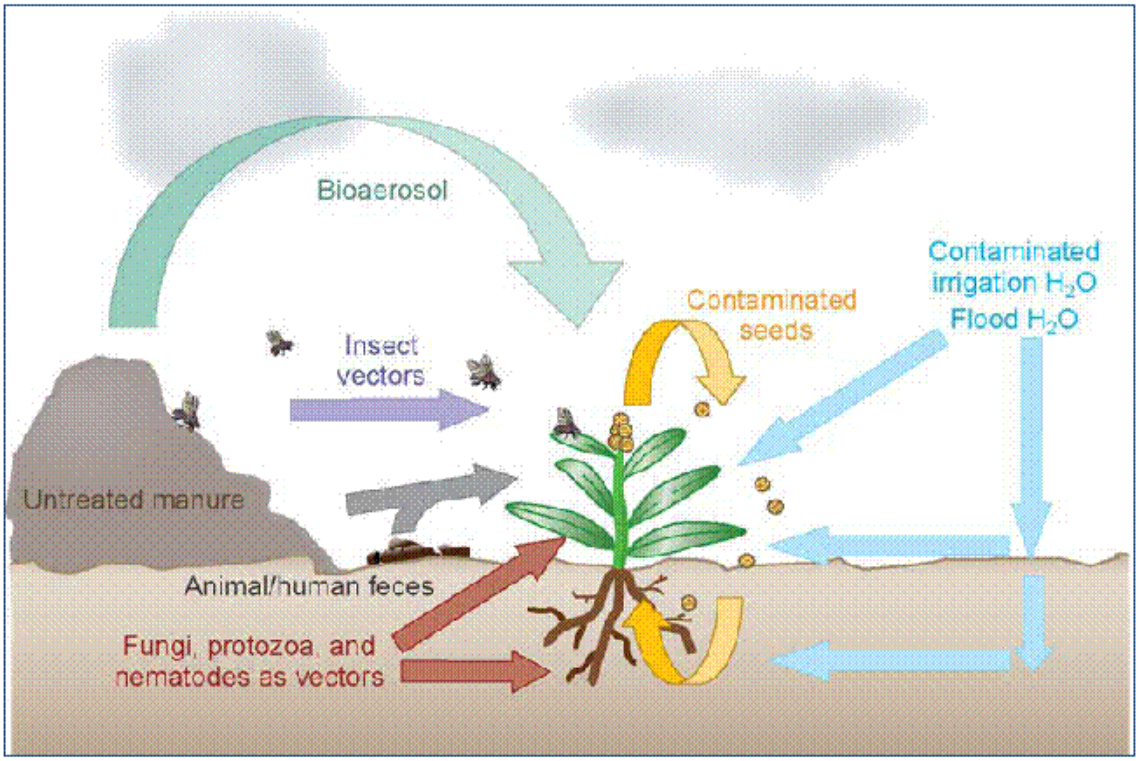

Figure 3.6. Factors affecting the contamination of vegetables with human pathogens in the field (Brandl, 2006).

To minimize the risks, the emphasis should be on preventing contamination by pathogens. The best way to prevent the introduction of pathogens into FC produces is by employing: Good Agricultural Practices (GAPs) for suppliers of raw materials, Good Manufacturing Practices (GMPs), developing and using Standards Operating Procedures (SOPs), and implementing an effective Hazard Analysis Critical Control Point (HACCP) plan. These programs work together to identify potential points of contamination and ensure that potential hazards are monitored and controlled to enhance safety (Harris *et al.*, 2002; Artés, 2004).

The U.S. Food and Drug Administration (FDA) issued the "Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables" in 1998 and it was recently supplemented by FDA's 2008 "Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables" (FDA 1998, 2008). These programs provide recommendations for food safety practices that are intended to minimize the microbiological hazards associated with fresh and FC produces. Similarly, AFHORLA issued the "Guía de Buenas Prácticas de Producción de IV Gama" in 2006 for the Spanish FC industry. Although, the use of these guides is not mandatory, its adoption by the industry is strongly recommended in order to minimize microbial food safety hazards and ensure food safety to consumers.

In Spain, the microbiological criteria for FC vegetables were referred in RD 3484/2000 (2001) which set out the standards of hygienic production, distribution, and marketing of food based on vegetables to be eaten raw. Recently, previous Spanish

legislation was repealed and RD 135/2010 (2010) was implemented on January 25, 2010. For this regulation, Spain has adopted the EU legislation (EC 1441/2007) on microbiological criteria for food, which is mandatory and has a direct application to the FC industry.

4. TOMATO POSTHARVEST HANDLING

4.1. Quality and maturity aspects

Tomato is a climacteric fruit vegetable with high respiration rate (10, 15, 22, 35 and 43 mg CO_2 /kg h at 5, 10, 15, 20 and 25°C respectively) and an ethylene emission rate of about 10 µL C_2H_2 /kg h at 20°C. Tomato is very sensitive to C_2H_4 with a threshold of 0.5 ppm. Physicochemical changes such as chlorophyll degradation, loss of firmness, slight decrease in acidity, and slight increase in soluble solids occur during the climacteric peak (Artés and Artés-Hernández, 2004).

Different maturity indexes have been developed to determine the optimum harvest date for tomatoes. These indexes normally include several aspects such as appearance, color, size, shape, and absence of physiological and pathological disorders. The external color is a good indicator of tomato ripening stage. There are commercial color charts to give objectivity to this important quality attribute and provide specific maturity and quality indexes to growers, packers, distributors, importers, and exporters (Artés and Artés Hernández, 2004). The USDA defines the ripening stages according to the following standards:

- (1) Green: Fruit surface is completely green; the shade of green may vary from light to dark.
- (2) Breaker: there is a definite break in color from green to tannish-yellow, pink or red on not more than 10% of the surface.
- (3) Turning: 10% to 30% of the surface is not green; in the aggregate, shows a definite change from green to tannish-yellow, pink, red, or a combination thereof.
- (4) Pink: 30% to 60% of the surface is not green; in the aggregate, shows pink or red color.
- (5) Light red: 60% to 90% of the surface is not green; in the aggregate, shows pinkish-red or red.
- (6) Red: More than 90% of the surface is not green; in the aggregate, shows red color.

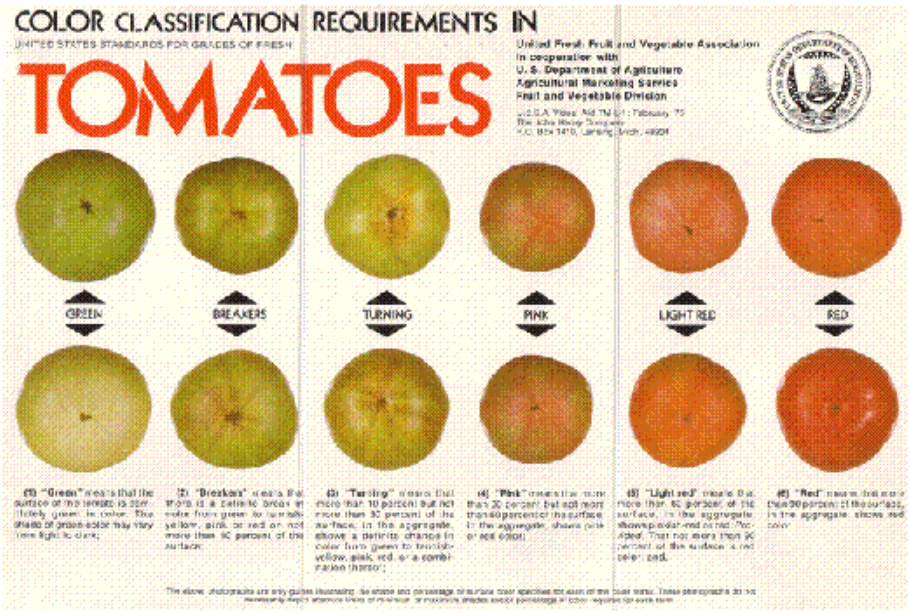

Figure. 4.1. Color classification requirements in tomatoes (USDA, 1991).

Each country has different specifications. In Europe, the tomato color chart Kleur-Stadia Tomaten created by Dutch Bureau for Horticultural Auctions is well known and currently in use.

Figure 4.2. Tomato color chart Kleur-Stadia Tomaten

Firmness is considered another important quality parameter for tomatoes. The table shown below lists the typical textural characteristics of tomatoes and their relationship to objective firmness (Cantwell and Kasmire, 2002).

Table 4.1. Typical textural characteristics of tomatoes and their relationship to objective firmness.

Firmness class	**Description based on resistance to finger pressure**	**Firmness* (mm compression)**
Very firm	Fruit yields only slightly to considerable pressure	0.5-1.0
Firm	Fruit yields only slightly to moderate pressure	1.0-1.5
Moderately firm	Fruit yields to moderate finger pressure	1.5-2.0
Moderately soft	Fruit yields readily to moderate finger pressure	2.0-2.5
Soft	Fruit yields to slight finger pressure	2.5-3.0
Very soft	Fruit yields very readily to slight finger pressure	> 3.0

*Measured by placing a 500 g weight for 10 seconds on the equator zone of the fruit

Many preharvest, harvest, and postharvest factors influence the composition and quality of tomatoes. These include inherent (genetic) and environmental factors such as climatic conditions (temperature, light, pollutants) and cultural practices (soil type, nutrient and water supply, use of agricultural chemicals, harvesting method). Maturity stage at harvest and postharvest handling procedures also affect tomato quality and its maintenance (Kader, 1986).

4.2. General processing and unit operations

The general flow chart of the process is showed below and based on Cantwell and Kasmire (2002) and Artés and Artés-Hernández (2004).

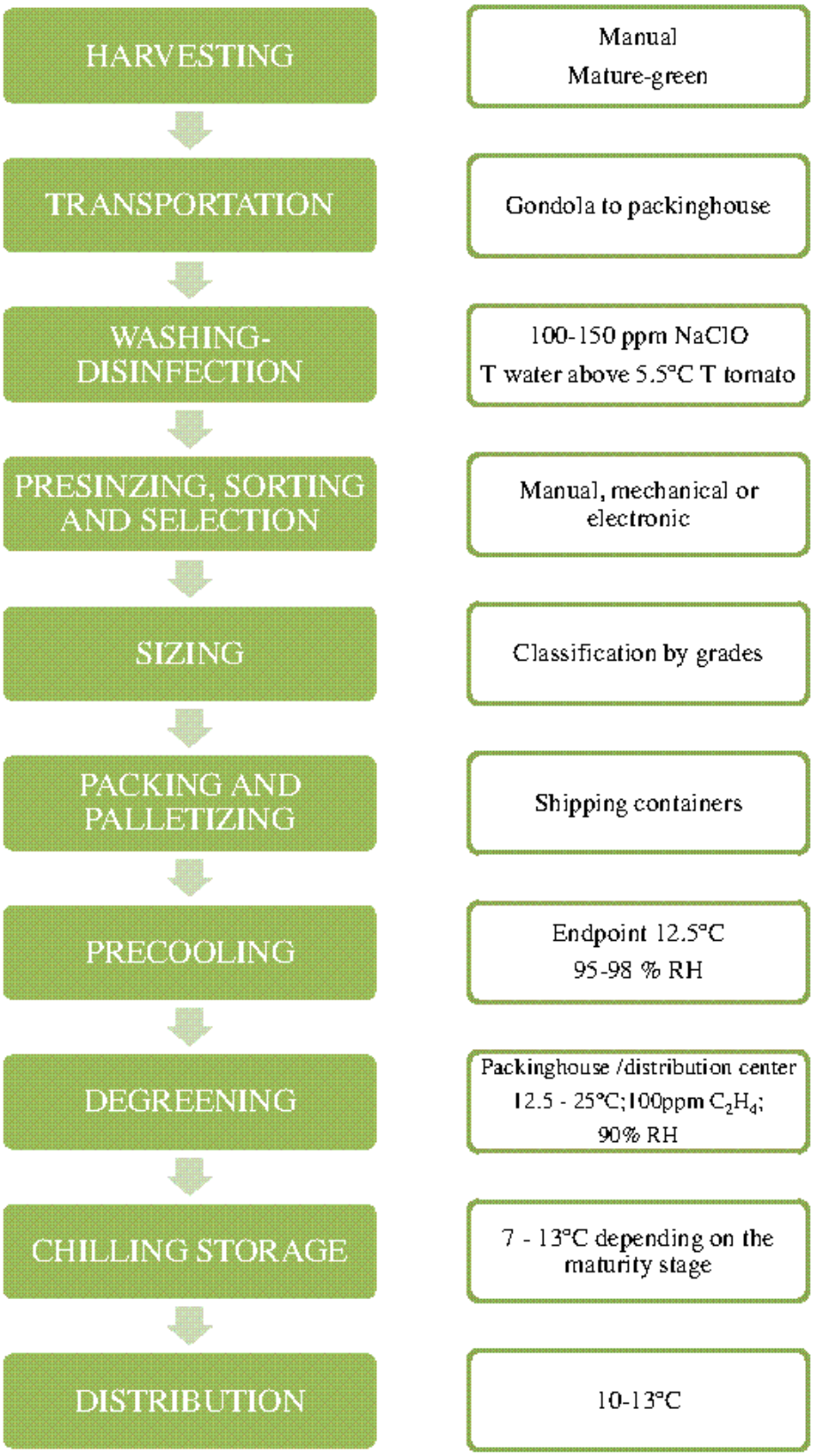

Figure 4.3. Unit operations of postharvest handling of mature-green tomatoes.

4.2.1. Harvest

Tomatoes can be harvested by hand or mechanically. Special care must be taken in all handling operations to prevent product damage and the associated loss of visual appearance, increase in water loss, and decay. Minimizing handling transfers of the tomatoes is the key factor for reducing mechanical damage.

4.2.2. Transportation

Harvested tomatoes are dumped into a trailer-mounted gondola and directly transported to the packinghouse, and held in a shade area until processing.

4.2.3. Washing-Disinfection

Tomatoes are frequently handled in packinghouses with re-circulated water systems, such as dump tanks and flume systems. These tanks are physically separated and the water temperature must be 5.5ºC above the pulp temperature to avoid infiltration of microorganisms. Water chlorination (150 mg/L NaClO, pH 6.5-7.5, contact time 2 mins max) is a common procedure for washing and disinfection of whole fresh tomatoes (Cantwell and Kasmire, 2002; Artés and Artés-Hernández, 2004).

4.2.4. Presizing, sorting, and selection

Tomatoes below a certain size are eliminated manually or mechanically by a presizing belt or chain. Undersized tomatoes are diverted to a cull conveyor or used for industrial processing. The purpose of the sorting step is to eliminate cull, overripe, misshapen, and otherwise defective fruit and separate tomatoes by color, maturity, and ripeness classes. Electronic color sorters are frequently used to separate mature-green and vine-ripe tomatoes or to classify them by different ripening stages. Tomatoes are sorted by quality according to grade standards depending on the country (Cantwell and Kasmire, 2002; Artés and Artés-Hernández, 2004).

4.2.5. Waxing

Edible waxes may be applied to tomatoes but this step is optional and scarcely used. Waxing replaces some of the natural waxes removed in the washing and cleaning operations, which reduces water loss and improves the appearance of the tomato. Fungicides may be added to the wax to avoid further postharvest disorders.

4.2.6. Sizing

After sorting for defects and color differences, tomatoes are segregated into several categories. Sizing may be done manually or mechanically by belt or weight sizers.

4.2.7. Packing and palletizing

Mature-green and pink tomatoes are commonly weighed or volume filled into shipping containers. Other options are to pack into shipping containers by count or bulk bins. Packed shipping containers of tomatoes in large volumes are palletized for shipment.

4.2.8. Quality control

As previously mentioned, each factory must have an implemented quality control system applied to the raw material, semi-processed and processed product, manufacturing processes, and the environment to guarantee the safety, suitability, and compliance of specifications. In addition, the company is required to have a procedure of product recall when the specifications are not met. Instrumentation equipments for quality control have to be calibrated frequently.

4.2.9. Precooling

Delays from harvest to cooling can result in excessive water loss. Thus, pre-cooling is an essential operation to minimize this risk. While conventional room-cooling is common, forced-air cooling is more uniform and produces a better quality fruit. The optimum RH must be around 95-98%. The pre-cooling endpoint should be about 12.5°C. New trends are focused on promoting a more efficient air circulation through the packages and increasing the contact with the fruit surfaces. The air speed should not exceed 4 m/s to avoid excessive fruit dehydration (Artés and Artés-Hernández, 2004).

4.2.10. Chilling storage and transport conditions

Mature-green tomatoes can be stored up to 14 days at 12.5°C prior to complementary accelerated ripening without significant reduction of sensory quality and color development. At this temperature, decay is likely to increase following storage beyond two weeks. Typically 8-10 days of shelf-life are attainable within the optimum temperature range after reaching the firm-ripe stage. Short term storage or transit temperatures below this range are used by some in the trade but will result in chilling injury after several days.

Tomatoes are chilling sensitive at temperatures below 8-10°C if held for longer than two weeks or at 5°C for longer than 6-8 days. Consequences of chilling injury are failure to ripe and develop full color and flavor, irregular (blotchy) color development, premature softening, surface pitting, browning of seeds, and increased decay (especially black mold caused by *Alternaria* spp.). Chilling injury is cumulative and may be initiated in the field prior to harvest (Cantwell and Kasmire, 2002).

The optimum RH should be around 90-95%, in order to maximize postharvest quality and prevent water loss (desiccation). Extended periods of higher humidity or

condensation may encourage the growth of stem-scar and surface molds (Cantwell and Kasmire, 2002; Artés and Artés-Hernández, 2004).

4.2.11. Accelerated ripening

For uniform and controlled ripening, ethylene is often applied to mature-green tomatoes. Satisfactory ripening occurs at 12.5°C to 25°C; the higher the temperature, the faster the rate of ripening. Above 30°C, red color development (due to lycopene synthesis) of the tomato is delayed due to inhibition of C_2H_4 production. Fruit ripened above 25°C will be less firm than those ripened at 15 to 20°C. A C_2H_4 concentration of about 100 ppm is commonly used. Tomatoes are usually held at 20°C with 90% RH and treated for up to three days. If tomatoes are at the minimum color stage of "breaker" (according to USDA color chart), a C_2H_4 treatment will not further accelerate ripening. The treatment is not necessary because the fruit is naturally producing its own C_2H_4 (Báez *et al.*, 1998; Cantwell and Kasmire, 2002, Artés and Artés-Hernández, 2004).

Ethylene treatments may be done at the shipping point or at destination markets. However, ripening uniformity and final fruit quality are generally considered best if the C_2H_4 treatment is applied at the shipping point soon after harvest. Tomatoes may be treated before or after packing, but most are treated after packing. An advantage of treating before packing is that the warmer conditions favor development of any decay on the fruit, so infected fruit can be eliminated before the final packing (Cantwell and Kasmire, 2002).

4.3. Physiological disorders

Blotchy ripening is a physiological disorder characterized by the randomized development of green or green-yellowish areas on the surface of red tomato fruit. Apparently, the development of this disorder is related to the availability of potassium and inorganic nitrogen in the soil system. Areas showing blotchy ripening have less organic acids, soluble solids content (SSC), and starch (Moretti *et al.*, 2000). Sunburn is associated with excessive exposure to sunlight, which causes elevated tissue temperatures during fruit development. This disrupts lycopene synthesis, which results in the appearance of yellow areas in the affected tissues that remain during the ripening process. Blossom-end rot is a physiological disorder involving a calcium deficiency that is either due to poor uptake or translocation into the fruit. Symptoms begin in the green fruit as a small discoloration at the blossom end that increases in size and becomes dry and dark-brown. Occurrence increases dramatically when calcium levels in the soil system drop below 0.08% (Moretti *et al.*, 2000). Eventually, secondary decay organisms colonize weakened tissues. Graywall is noticeable as necrotic vascular tissue in the pericarp fruit wall. The graywall begins developing at the green stage and has been associated with marginal growing conditions such as cool weather, low light levels, poor nutrition, saturated soils, tobacco mosaic virus, and bacteria; however, the cause is still undetermined. Graywall can be a serious disorder in both field and greenhouse

production systems (Jones *et al.*, 1999). Irregular ripening is characterized by the appearance of non-uniform ripening and white internal tissue. It has been associated with the feeding of sweet potato whitefly (*Bemisia argentifolii*) on tomato fruit (Hanif-Khan *et al.*, 1997). Internal bruising is recognized by the appearance of yellow to green locular gel in ripe tomatoes. It is caused by an impairment of normal ripening of the locular gel following a physical impact at the green or breaker stage of ripeness (MacLeod *et al.*, 1976). Fruit with internal bruising show significant reductions in vitamin C content, titratable acidity (TA), consistency, and total carotenoids content (Moretti *et al.*, 1998). Besides altering quality attributes, internal bruising also affects tomato fruit flavor (Moretti *et al.*, 2002). Breaker-stage tomatoes are more sensitive to internal bruising than those handled at the green stage (Sargent *et al.*, 1992).

4.4. Pathological disorders

Tomatoes are susceptible to numerous fruit decays from the fields through postharvest handling. Postharvest decays often develop in wounds, bruised tissue, and during fruit softening. Tomatoes can be infested by plant pathogens via cross-contamination from diseased fruits, dirty harvest containers, and from poorly sanitized water handling systems, and packing line components. Populations of decay pathogens can be adequately controlled through a regular sanitation program in the field and during handling, packing, and ripening/storage operations. Bacterial decays include soft rots (*Bacillus* spp., *Erwinia carotovora* spp., *Pseudomonas* spp., and *Xanthomonas campestris*) and lactic acid decay commonly known as bacterial sour rot (*Lactobacillus* spp. and *Leuconostoc mesenteroides*) (Conn *et al.*, 1995; Bartz *et al.*, 1995). Fungal decays include Alternaria rot or Black rot (*Alternaria alternata*), Fusarium rot (*Fusarium* spp.), Gray Mold rot (*Botrytis cinerea*), Mucor rot (*Mucor mucedo*), Phoma rot (*Phoma* spp.), Phomopsis rot (*Diaporthe* spp.), Phytophthora rot (Buckeye rot) (*Phytophthora* spp.), Pleospora rot (*Pleospora herbarum*; *Stemphylium botryosum*), Rhizopus rot (*Rhizopus stolonifer*, *R. oryzae*), Ring rot (*Myrothecium roridum*), Sclerotium rot (*Sclerotium rolfsii*), Sour rot (*Geotrichum candidum*), Target spot (*Corynespora cassiicola*), and Watery soft rot (*Sclerotinia minor, S. sclerotiorum*) (Jones *et al.*, 1991; Snowdon, 1992).

4.5. Safety aspects of fresh market tomatoes

Tomatoes have been frequently associated with outbreaks of human salmonellosis in the last years. Diverse *Salmonella enterica* serotypes have been implicated in these infections, including Newport, Typhimurium, Javiana, Anatum, Thompson, and Muenchen (CDC 2005, 2007). Recently, a *S. enterica* sv. SaintPaul outbreak related with tomato and Jalapeño and Serrano peppers involved more than 1,400 cases from the U.S. and Canada (CDC, 2008). In order to minimize the microbiological hazards associated with fresh and FC tomato products the United Fresh Produce Association in collaboration with the North American Tomato Trade Work Group (NATTWG) published the "Commodity Specific Food Safety Guidelines for the

Fresh Tomato Supply Chain" in 2006 and then updated it in 2008. These programs provide recommendations for food safety practices that are intended to minimize the microbiological hazards associated with fresh and FC tomato products during growing, harvesting, transportation, cooling, packing, and storage.

Tomatoes are frequently handled in packinghouses with some systems of re-circulated water, such as flume systems and dump tanks. The water used in these systems can become infested with pathogens, including plant pathogens, bacteria, and human pathogens, which can cause tomato decay and even human foodborne outbreaks. The use of sanitizing agents during the washing step is essential to avoid cross contaminations.

Since tomatoes are a warm-season crop, high pulp temperatures can be reached during harvesting and transporting operations to the packinghouse. Internalization of bacteria into the stem scar has been demonstrated with tomatoes submerged in water that is cooler in temperature than the pulp of the tomatoes (Bartz, 1981, 1982). When tomatoes cool in the water system, a vacuum is created which can cause water infiltration and potential pathogens to be drawn into the pores of the tomatoes. Therefore, water temperature, pulp temperature, and water quality are critical considerations for maintaining the safety and quality of the product. In general, cold water immersion as a cooling technique shall not be used. If a dump tank is used, the water temperature must be 5.5ºC higher than the incoming fruit pulp temperature to minimize the risk of intrusion of microorganisms into the tomatoes (Bartz 1988; Zhuang *et al.*, 1995; Ibarra-Sánchez *et al.*, 2004).

5. SANITATION OF FRESH-CUT FRUIT AND VEGETABLES: NEW TRENDS, METHODS AND IMPACTS

Extracted from: Artés, F., Gómez, P.A., Tomás-Callejas, A., Artés-Hernández, F., 2011. Sanitation of fresh-cut fruit and vegetables: New trends, methods and impacts. In: McMann, J.M. (Ed), Potable water and sanitation. Nova Science Publishers, Hauppauge, New York, USA. pp. 1-36.

5.1. Introduction

Consumers are more and more conscious about the relationship between the food they eat and their overall health and well-being. This fact has increased the demand of convenient fresh food, keeping their living fresh state, free from additives, with high nutritional value and powerful antioxidant properties which fight reactive oxygen species. The industry satisfies this particular demand by offering FC fruit and vegetables. FC produces are elaborated free from additives by using light combined methods such as washing, cutting, disinfecting, and packaged at chilling temperatures under polymeric films able to generate optimum MAP conditions (Artés and Allende, 2005). FC commodities usually need no further processing prior to consumption, offering important advantages for consumers since, in addition to convenience and functionality, they have high quality and any or low wastage at a reasonable price (Wiley, 1994; Beuchat, 2002; Bruhn, 2002; Artés, 2004).

Despite the benefits derived from eating raw fruits and vegetables, due to their specific methods of preparation, FC plant foods are highly perishable and must be elaborated following strict control procedures for reducing overall quality loss and assuring its safety to consumers. FC processing of plant produce stimulates microbiological growth, which may be potentially harmful to human health. Due to this, safety is an issue of concern as these foods have long been known to be vehicles for transmitting infectious diseases (Leistner and Gould, 2002; Artés and Allende, 2005).

FC produces are alive even when processing destroys plant tissues increasing metabolic activity and inducing senescence and loss of resistance to microbial spoilage. Plant damage stimulates respiration rate (RR), C_2H_4 emission, enzymatic and non-enzymatic colour change and nutrient loss from cells. These changes lead to quality loss and reduced shelf-life when compared to that found for the intact product (Wiley, 1994; Artés *et al.*, 2007). There are also pre-harvest factors like plant *cv*, growing conditions, harvest time and maturity/ripening stage at harvest that have a high influence on postharvest life. Processing conditions (precooling, trimming, cleaning, conditioning, cutting, peeling, coring, handling, washing, disinfecting, draining, rinsing, drying, packaging) and distribution conditions (temperature, RH , atmosphere composition and duration) highly determine produce characteristics. For being successful, FC industry must be seen as a highly integrated system with all processing steps considered in combination with the others (Artés, 2004).

FC industry is really concerned about fresh-like quality, safety and high nutritional value. Moreover, it is more and more oriented to look for sustainable procedures, especially those related to sanitation. The main preservation technique to prevent or delay spoilage is chilling storage combined to active or passive MAP. Also some chemical coadjutants like antimicrobial solutions, acidulants and antioxidants are commonly used (Leistner and Gould, 2002, Artés and Allende, 2005).

This work intends to review the major sanitation approaches which can be used for keeping quality and safety of FC plant commodities within a sustainable frame represented by antimicrobial solutions, electrolyzed water (EW), O_3, ultraviolet (UV-C) light, intense light pulses, and innovative MAP under superatmospheric O_2 and noble gases.

5.2. Antimicrobial agents applied as solutions

In many situations of the FC industry, chlorination of water is one of the primary elements of a properly managed postharvest sanitation program. This operation is considered as the most important to disinfect water and minimize the transmission of pathogens from infested plant produce, water or debris, to non-infested surfaces such as those mechanically injured during harvesting, transportation or processing, wounds, or the natural plant surface openings (Artés *et al.*, 2009). Disinfectant capacity of NaClO is related with its very high oxidizing properties making it useful for sanitizing both products and equipment of the processing area (Nieuwenhuijsen *et al.*, 2000). It is effective for most situations, relatively inexpensive, and useful in operations of any size (Suslow, 1997). Effectiveness of NaClO against microorganisms mainly depends on concentration since their efficacy increases when increasing concentration, but also on pH, temperature, organic matter present in the washing water and plant commodity, time of exposure, and initial microbial load of raw material (Boyette *et al.*, 1993; Gómez *et al.*, 2010). However, very high NaClO levels may cause product tainting (Adams *et al.*, 1989) and sodium residue on the product and equipments (Ritenour and Crisosto, 1996).

NaClO in contact with water induces an increase in pH and generates hypochlorous acid (HOCl), which is the active antimicrobial compound. The acid dissociates readily to hypochlorite ions (OCl^-) at high pH, or chlorine gas (Cl_2) at low pH, thus the pH must be kept in the range of 6.5 to 7.5 for HOCl to be stable and efficient (Boyette *et al.*, 1993; Suslow, 1997). It should be indicated that, even if NaClO is more efficient at low pH levels, values between 6 and 7.5 should be selected for reducing the risk of corrosion of metallic processing equipment (Beuchat, 2000).

An understanding of NaClO chemistry together with a definition of terms is necessary to describe the amount of chlorine in any form available for oxidative reaction and disinfection. Total chlorine refers to the sum of free and combined chlorine in solution. Free chlorine, free available and free residual chlorine refer to chlorine in

the form of elemental chlorine (Cl_2), hypochlorous acid (HClO) and hypochlorite ion (OCl^-) (Simons and Sanguansri, 1997).

NaClO may partially oxidize food constituents originating unhealthy by-products, such as chloroform ($CHCl_3$), haloacetic acids or other trihalomethanes (THM) that have known or suspected carcinogenic or mutagenic potential effect, with proved toxicity to liver and kidney (Nieuwenhuijsen *et al.*, 2000; Hrudey, 2009; Ölmez and Kretzschmar, 2009). At the same time, at pH over 7.5 NaClO reacts with organic N-based materials to produce chloramines (Suslow, 1997). These negative aspects related with chlorine have induced some European countries, like Germany, The Netherlands, Denmark, Switzerland and Belgium to forbid the use of NaClO for disinfection of FC produces (Betts and Everis, 2005; Carlin and Nguyen-the, 1999). An option to reduce THM levels is to change chlorine by chloramine disinfection because they do not react with organic matter in the water to form THM. Adding ammonia to a chlorination system switches NaClO to chloramines. However chloramines could be considered as a rather less acceptable alternative because its efficacy as a disinfection agent is lower and could be risky for workers organs like eyes and respiratory tract.

It has been demonstrated that effectiveness of NaClO is limited to some products (Beuchat and Brackett, 1990). Actually, chlorine compounds are helpful in reducing the aerobic microbial counts in many leafy vegetables, but not necessarily in root vegetables. At the same time, it was reported that NaClO is not very effective for inhibiting *L. monocytogenes* growth in shredded lettuce or Chinese cabbage (Ahvenainen, 1996).

According with previously mentioned concerns, considerable research is nowadays done looking for alternatives to NaClO. Among them, some organic acids formulations, such as peroxyacetic acid (PA) combined with citric and ascorbic acids, ClO_2 and H_2O_2, calcium lactate, EW, steamer jet injections and biological compounds have been tested with varying results as it will be described below.

5.2.1. Peroxiacetic acid

PA is a combination of peracetic acid (CH_3CO_3H) and H_2O_2, and typically commercialized as a liquid. PA is an interesting alternative to NaClO since its break-down products, acetic acid, O_2, CO_2, and water makes its use completely sustainable and eco-friendly. Its first application was for cleaning surfaces in concentrations ranging from 85 to 300 ppm. The FDA of the USA has set a minimum of 85 ppm PA for cleaning hard surfaces where food is handled (FDA, 1997), although for cleaning food's surface 50 ppm is commonly enough (Stampi *et al.*, 2001) while levels used in the environmental and medical fields normally ranged from 1,200 to 2,600 ppm.

Related to the mechanism of action of PA, it has been suggested to act primarily on lipoproteins in the cell membrane, and it may be possible that it is equally effective

against outer membranes lipoproteins, facilitating its action against Gram-negative cells (Leaper, 1984).

PA tolerates several adverse factors like a wide range of temperature application, pH changes (from 1 to 8), water solutions with high carbonate concentration and soil contaminated. By these positive sides, its current main area of application is in fruit and vegetables processing (Artés *et al.*, 2009). For plant surfaces treatment, recommended formulations combine H_2O_2 11% and CH_3CO_3H 15%, at 80 ppm, followed by rinsing with tap water (Suslow, 1997). It has been reported that it was effective for controlling *E. coli*, *Salmonella* spp., and *L. monocytogenes* in FC products (Park and Beuchat, 1999; Rodgers *et al.*, 2004; Ruiz-Cruz *et al.*, 2007). In *Enterobacter sakazakii* counts decreased 5 log units in lettuce with PA applications (Kim *et al.*, 2006). Compared to 150 ppm NaClO, 80 ppm PA reduced the psychrotrophic counts by 2 log units and mesophilic counts by 1 log unit in FC Galia melon, resulting in the pieces having a shelf-life of 10 days at 5°C. However, this sanitizer decreased the total vitamin C and the antioxidant activity (Silveira *et al.*, 2010). Vandekinderen *et al.*, (2009) studied the impact of a decontamination step on the shelf-life, sensory quality and nutrient content of grated carrots under MAP and stored at 7°C. 80 mg/L PA showed possibilities for extending shelf-life without pronounced effects on nutritional content.

5.2.3. Chlorine dioxide

ClO_2 is a stable dissolved gas with a strong oxidative power and penetration, higher than that found for NaClO (FDA, 1998). ClO_2 is a strong bactericide and virucide at levels as low as 0.1 ppm having also a very high effectiveness against spores. It attacks planktonic and sessile bacteria and fungi, disinfects surfaces, and prevents and removes rapidly bio films, avoiding bacterial re-growth (EPA, 1999). ClO_2 does not need a long contact time for being highly effective against pathogenic organisms such as *Legionella*, amoebal cysts, *Giardia cysts*, *E. coli*, and *Cryptosporidium* (Xie, 2003). Different compounds of bacterial cells membrane react with ClO_2, causing the interruption of several cellular processes. Cell membrane has been identified as the primary target of ClO_2 on microbial cells but ClO_2 also reacts directly with amino acids and the RNA attacking the cell structure and/or the acids inside the cell. The production of proteins is prevented and the cell membrane affected by changing membrane proteins and lipids (EPA, 1999).

Viruses are eliminated in a different way than bacteria. ClO_2 reacts with peptone, a water-soluble substance that originates from hydrolysis of proteins to amino acids, and kills viruses by prevention of protein formation being more effective than NaClO or O_3 (EPA, 1999).

Differently to NaClO and NaBrO, ClO_2 does not ionize to form weak acids neither to form carcinogenic by-products, like THM. This allows ClO_2 to be effective over a wide pH range. However, differently than PA, which is not dangerous, ClO_2 and

its disinfection by-products (chlorite and chlorate) can create problems for dialysed patients (EPA, 1999).

ClO_2 can be used as both gas and aqueous type. As a gas it has more penetrability than the aqueous sanitizers (Han *et al.*, 2001). Different factors such us gas concentration (0.1-0.5 mg/L), time (7-135 min), RH (55-95%) and temperature (5-25ºC) affect the antimicrobial effect of ClO_2 gas on the inactivation of *E. coli* O157:H7 (Han *et al.*, 2001; Gómez-López *et al.*, 2009). One of the most important qualities of ClO_2 is its high water solubility, especially in cold water, which makes it strongly recommended for using in the FC industry. ClO_2 remains as dissolved gas in solution being approximately 10 times more soluble in water than NaClO.

Main ClO_2 drawback is that it must be generated on-site by reacting sodium chlorite and acid or NaClO (EPA, 1999). Today, new technology allows a more easy production, like systems where the reactants are packaged in pouches for reacting together. However, ClO_2 is unstable and can be explosive when concentrations reach 9.5% or more in air (Betts and Everis, 2005; Jin *et al.*, 2009).

While for drinking water less than 1 ppm ClO_2 is allowed, for whole produce a maximum concentration of 3 ppm is permitted (EPA, 1999; CFR, 2007a,b). The effect of ClO_2 to inhibit the main foodborne pathogens related with the FC commodities has been reported previously. Concentrations of 4-5 mg/L with contact times of 6 – 30 min were effective to reduce *Salmonella* spp., *E. coli* O157:H7 and *L. monocytogenes* inoculated onto cabbage, carrot, lettuce, strawberry and melon (Sy *et al.*, 2005; Mahmoud *et al.*, 2007; Mahmoud and Linton 2008a,b).

A solution of 5 ppm ClO_2 has been effectively used for apple, lettuce, strawberry and cantaloupe for inhibiting inoculated *E. coli* and *L. monocytogenes* (Rodgers *et al.*, 2004). In FC faba bean (*Vicia faba* L) the immersion for 2 min in 4 ppm ClO_2 was as effective as 150 ppm NaClO + 1g/L citric acid in reducing enterobacteria and mesophilic counts after 8 d at 5ºC, decreasing browning and improving sensory quality (Artés *et al.*, 2007; 2009).

Although the lack of knowledge about toxicity of liquid ClO_2 disinfection by-products make further studies necessary to establish the suitability of this chemical for FC produce sanitation. López-Gálvez *et al.* (2010) demonstrated that the use of aqueous 3 mg/L ClO_2 was equally effective as 100 mg/L NaClO and it did not cause any detrimental effect on the sensory and nutritional quality of FC Iceberg lettuce without the potential formation of THMs. Tomás-Callejas *et al.* (2010) evaluated *Salmonella* spp. removal and cross-contamination potential during the washing-disinfection, rinsing, and de-watering steps in FC baby Red Chard. Typical industry rates of 20 ppm NaClO (at pH 7.0) and 3 ppm ClO_2 substantially prevented cross contamination by *Salmonella* among co-mingled Red Chard leaves but neither fully disinfected the pathogen from inoculated leaves.

5.2.4. Hydrogen peroxide

H_2O_2 is a strong oxidising agent that is commonly used in the industry and in the medical field. It is considered a powerful bactericide, being effective even against spores. It is unstable and decomposed upon standing, agitation, and exposure to light or heating, producing water and O_2. However, H_2O_2 is also able to generate other cytotoxic oxidising species like hydroxyl radicals (Khadre and Yousef, 2001). A efficacy of H_2O_2 washing, similar to that of NaClO, has been demonstrated in extending shelf-life and reducing native microbial and pathogen populations, including *E. coli*, in whole grape, prune, apple, orange, mushrooms, melon, tomato, red bell pepper and lettuce, and in FC cucumber, zucchini, bell peppers, and melons (Sapers, 2003; Artés *et al.*, 2009). H_2O_2 is not currently approved as a sanitizing agent for fresh produce even when is permitted for other uses in food processing and packaging because it leaves no potentially harmful residues and it has been approved by FDA.

For disinfection of FC commodities, the use of dilute H_2O_2 solution has shown to be promising. For example, washing with 5% H_2O_2 was more effective than with 1000 ppm NaClO and Na_3PO_4 for reducing the microbial load on cantaloupe rinds, thus improving microbial quality and shelf-life (Sapers and Simmons, 1998). Residual H_2O_2 in treated fruits and vegetables might be eliminated passively by the action of endogenous catalase, given enough time for reaction, or actively by rinsing immediately after treatment to avoid reactions between H_2O_2 and food constituents that might affect product quality or safety. Ukuku (2004) studied the effect of H_2O_2 on microbial quality and appearance of whole and FC melons contaminated with *Salmonella* spp. H_2O_2 treatments of whole melon (2.5% and 5%) for 5 min caused a 3 log CFU cm^{-2} reduction of the indigenous surface microflora and 3 log CFU cm^{-2} reduction in *Salmonella* in all melon surfaces. However, browning of shredded lettuce increased after dipping in a H_2O_2 solution (Parish *et al.*, 2003).

Pedahzur *et al.* (1997) noticed an increase in the bactericidal effect of H_2O_2 and silver on *E. coli* when applied together, suggesting that such increase in the bactericidal effect of these two disinfectants might be due to the synergism between the two chemicals. Gopal *et al.* (2010) examined the use of silver and H_2O_2 as possible alternative to NaClO. Combination of electrochemically generated silver (5 ppm) and H_2O_2 (0.4 ppm) caused a reduction in the total plate count (0.87 log), *Pseudomonas* (2.66 log), *Enterobactericeaea* (1.61 log) and yeast and mould (1.60 log) immediately after washing in comparison to water-washed shredded lettuce. The combination of 1% H_2O_2 with 25 µg/mL nisin, 1% sodium lactate, and 0.5% citric acid (HPLNC) for reducing transfer of bacterial pathogens from whole melon surfaces to FC pieces has been tested. HPLNC reduced the number of *E. coli* O157:H7 and *L. monocytogenes* by 3 to 4 log CFU cm^{-2} on melon and the natural microflora on FC melons were also substantially reduced (Ukuku *et al.*, 2005).

The use of H_2O_2 as vapour instead of water solutions also appears to reduce microbial counts, extending shelf-life and maintaining quality of FC green bell pepper, cucumber, and zucchini (Sapers, 2003).

5.2.5. Citric acid Ascorbic acid Calcium

Organic acids and calcium have been mostly applied for controlling enzymatic and non-enzymatic browning in many fruit and vegetables (Sapers, 1993). They have also a positive effect for avoiding texture deterioration (Rosen and Kader, 1989) and microbial growth (Yildiz, 1994) at levels that did not adversely affect taste and flavour of plant commodities. Organic acids and calcium are more effective for bacteria than for moulds and yeast due to the low pH (between 2.1 and 2.7) at which they are applied.

Studies on FC Chinese cabbage showed that citric acid (10 g/L) repressed the petiole sprouting (black speck) development and prolonged their shelf-life from 10 days (control) to 14 days at 5°C. When stored at 0°C the shelf-life was not extended by citric acid, ascorbic acid and $CaCl_2$ dips, but citric acid improved quality by reducing black speck. In this experiment, treatment with citric acid produced only a slight pH reduction without affecting taste. The $CaCl_2$ (10 g/L) was beneficial in reducing browning while no microbial spoilage occurred after 35 d at 0°C or 21 d at 5°C under any treatment (Kim and Klieber, 1997).

FC Amarillo melon dipped in 0.52 mM citric acid for 30 s before MAP reached a shelf-life of 10 days at 5°C. This treatment kept microbial safety and avoided translucency and discoloration. Compared to 1.4 mM NaClO, citric acid increased lightness and improved visual appearance of melon pieces (Aguayo *et al.*, 2003). Dipping green celery crescents in a 0.5 M ascorbic and 0.1 M citric acid solution was as effective as 100 mg/L NaClO for reducing microbial counts and improving consumers acceptability (Gómez and Artés, 2004). Ölmez and Temur (2010) investigated the effects of 0.25 g/100 g citric acid + 0.50 g/100 g ascorbic acid solution for 2 min at 10°C on biofilms and attachment of *E. coli* and *L. monocytogenes* on green leaf lettuce. The treatment was unable to efficiently reduce or detach bacterial cells inside the biofilms or those cells attached to inaccessible sites. Moreover, a better understanding of the mechanism involved in bacterial attachment and biofilm formation is needed.

Cell wall structure and firmness of plant commodities is related to calcium since it maintains them by combining with pectin to form calcium pectate. Moreover, calcium lactate avoids the off-flavours associated with chloride salt, being a good alternative to it (Luna-Guzmán and Barret, 2000). Studies with FC melon showed that treatment with calcium generally kept firmness better than control during storage (Aguayo *et al.*, 2007; Luna-Guzmán *et al.*, 1999). FC lettuce seems also to be favoured when treated with 120 ppm NaClO and 15 g/L calcium lactate at 20 and 50°C. Samples washed with calcium lactate showed higher crispness than those immersed in NaClO even when lower than those which received a heat shock treatment (50°C). Authors (Martín-Diana *et al.*,

2006a) hypothesised that calcium lactate avoided the loss of turgor during the first days of storage while the combination with 50ºC extended this benefit to 12 days. It is possible that it may occur the activation of texture-related enzymes, as the pectin methylesterase, affecting the washing solution absorption into the lettuce. This might in turn increase the amount of calcium retained by the vegetable (Martín-Diana *et al.*, 2006a). Experiments with FC carrots showed that washing with 15 g/L calcium lactate at 50ºC was effective in reducing lignification at the cutting-edge area while keeping turgor of cortex tissue cells. Data about microbial growth should be given in order to a better knowledge of the effects (Rico *et al.*, 2007). In that way, Aguayo *et al.* (2007) found that $CaCl_2$ and calcium lactate reduced the bacterial growth by 2 log CFU/g and the yeast growth by 1 log CFU/g when diced melon was stored up to 8 days at 5ºC. Moreover, FC melon dipped into hot calcium solution (60ºC, 1 min) as $CaCl_2$ (0.5%), or weak organic acid salts like calcium propionate (0.9%) or lactate (1.4%) had a lower microbial growth and a better firmness. This could be explained by an increment in bound calcium concentration (50%) which in turns produces firmer melon pieces.

5.2.6. Electrolyzed water

EW is generated by electrolysis of a dilute salt (NaCl, usually about 0.1%) solution of pure water in an electrolysis chamber where anode and cathode electrodes are separated by a membrane. On the anode side, acidic EW is generated and has strong bactericidal effect on most known pathogenic bacteria, due to its low pH, high oxidation–reduction potential (ORP) (about 1100 mV) and the presence of hypochlorous acid. The cathode area produces alkaline reducing water (Ongeng *et al.*, 2006).

The acidic type was the first form developed and accepted by the food industry in Japan (Izumi, 1999). It was found to be useful at killing bacteria and parasites on raw fish without altering its sensory characteristics. EW has a strong bactericidal effect against pathogens and spoilage microorganisms, more effective than NaClO due to its high redox potential (Izumi, 1999; Koseki and Itoh, 2001; Bari *et al.*, 2003). Hypochlorous acid is present in EW at pH 6.8 and it is generated by electrolysis of NaCl solution, since HCl formed at the anode site neutralizes the NaOH at the cathode site. Advantages of using EW at neutral pH are that it does not affect pH, surface colour or general appearance of FC vegetables (Izumi, 1999).

In the recent years, several studies have reported that the use of EW in FC fruit and vegetables is greatly influenced by type of vegetable and minimal processing. (Izumi, 1999; Koseki and Itoh, 2001; Wang *et al.*, 2004; Ongeng *et al.*, 2006, Rico *et al.*, 2008). EW containing 15 to 50 ppm available NaClO was effective as a disinfectant for FC carrots, spinach, bell pepper, potato and cucumber, without discoloration and lowering microbial counts from 0.6 to 2.6 log units (Izumi, 1999).

Acetic EW disinfected the surfaces of lettuce by reducing the aerobic and coliform bacteria, moulds, and yeasts, with a similar effect of NaClO, resulting in a 1 log unit reduction for total aerobic counts and 0.5 log for coliforms in shredded lettuce pieces. After 7 days at 1-2°C, physical and sensory parameters did not change (Wang *et al.*, 2004). In other study, it was reported that EW has no impact on taste, smell, and texture of shredded lettuce (Koseki *et al.*, 2001). In the same way lettuce samples washed with EW containing 60 mg/L free chlorine (pH 6.5) resulted as effective as NaClO, showing a good quality retention (Rico *et al.*, 2007a). Growth of *Salmonella* was reduced in alfalfa seeds and sprouts when treated with EW (Kim *et al.*, 2003) while acetic EW has also been shown to effectively inactivate *E. coli* O157:H7, *S. enteritidis* and *L. monocytogenes* on lettuce (Park *et al.*, 2001).

The use of acidic EW (AEW) and neutral EW (NEW) as disinfectant for food processing equipment has been studied (Venkitanarayanan *et al.*, 1999a; Kim *et al.*, 2001; Park *et al.*, 2002; Guentzel *et al.*, 2007). Ayebah and Hung (2005) indicated that AEW did not have any adverse effect on stainless steel. However, issues such us gas emission, strong acidity of AEW, metal corrosion, free chlorine content and by-products formation need to be supported for further research.

The efficacy of NEW and AEW to reduce natural microflora as well as the main foodborne pathogens associated to FC produce has been demonstrated. Using AEW alone resulted in moderate control of bacterial growth of FC cilantro during storage (Wang *et al.*, 2004). Reductions of viable aerobes by 2 log CFU/g were obtained in lettuce washed with AEW (pH 2.6; oxidation reduction potential, 1140 mV; 30 ppm of available chlorine) for 10 min (Koseki *et al.*, 2001). Also, AEW was effective to reduce *E. coli* O157:H7 (Venkitanarayanan *et al.*, 1999b; Sharma and Demirci, 2003; Liao *et al.*, 2007; Stopforth *et al.*, 2007), *Salmonella* and *L. monocytogenes* (Venkitanarayanan *et al.*, 1999b; Fabrizio and Cutter, 2003, Stopforth *et al.*, 2007). Due to its neutral pH, NEW could be less aggressive to the corrosion of processing equipments compared to AEW. NEW (pH 6.8) containing 15 to 50 ppm available chlorine was effective as a disinfectant for FC vegetables without causing discoloration. NEW did not affect tissue pH, surface colour or general appearance (Izumi, 1999). Abadías *et al.* (2008) demonstrated that the bactericidal activity of diluted NEW (50 ppm of free chlorine, pH 8.60) against *E. coli* O157.H7, *Salmonella*, *L. innocua* and *E. carotovora* on FC lettuce was similar to that of 120 ppm free NaClO.

A theory for the inactivation mechanism of bacteria by EW proposed that ORP could firstly affect and damage the redox state of glutathione disulfide-glutathione couple, and then penetrate the outer and inner membranes of *E. coli* O157:H7, resulting the necrosis (Liao *et al.*, 2007).

The application of EW in the food industry has been recently reviewed by Huang *et al.* (2008) as a disinfectant for food processing equipment, vegetables, fruits, poultry, meat and seafood. However, there is scarce information about the effects of

NEW and AEW on the main quality attributes in FC vegetables. Martínez-Hernández *et al.*, (2009) showed that the use of AEW (40 ppm free chlorine, pH 3.0) retained the total content of polyphenols and chlorophylls better than a conventional 100 ppm NaClO treatment during the shelf-life of FC Mizuna baby leaves stored at 5ºC up to 11 days.

EW has many potential uses for the food industry being advantageous because it involves on-site production of the disinfectant, which means there are no chemicals to store or handling costs for dealing with them. The main problem is that, in some cases, it has a short shelf-life (about 2 weeks); therefore mostly only companies with access to an electrolysis machine could get profit from its benefits (Artés *et al.*, 2009).

5.2.7. Steamer jet injection

Vapour or hot water could be seen as an alternative to replace NaClO. However, exposure to high temperatures may produce detrimental changes in the product lowering its nutritional value. By that reason, exposure time to steamer jet injection should be low and much research is needed to optimise exposure conditions for FC plant commodities. In that way, Martín-Diana *et al.* (2007) reported a lower RR and a partial inactivation of browning-related enzymes when FC lettuce was exposed to water steam. Moreover, mesophilic counts were maintained as low as when NaClO was applied. Independently of these positive effects, ascorbic acid and carotenoids content showed a reduction, even when samples look like it did not, showing similar values of acceptability and fresh appearance than control.

5.2.8. Biological compounds, natural microbiota and/or their antimicrobial products

Nowadays efforts to reduce or eliminate chemically synthesized additives in foods are conducted to find natural alternatives to control bacterial and fungal growth in fresh-cut plant commodities (Soliva-Fortuny and Martín-Belloso, 2003). Commercial products based on natural biocide action such us lactate esters from lactic acid and alcohols (Purac), organic acids and flavonoids (Citrox), and lactoperoxidase, H_2O_2 and thiocyanate (Catallix) have been released to the market. Citrox and Purac applied at the commercial recommended doses did not prevent transfer of *E. coli* cells between inoculated and non inoculated FC lettuce and therefore indicating cross-contamination during washing (López-Gálvez *et al.*, 2009). Other natural derivate compounds such us grapefruit seed extracts (Xu *et al.*, 2009) and green tea extracts (Martín-Diana *et al.*, 2008) has been studied due to their antimicrobial effect in order to extend the shelf-life of FC commodities.

Eugenol (from clove, *Syzygium* sp.) and thymol (from thyme and oregano) exhibited antioxidant and antimicrobial effects on MAP stored table grape (Burt, 2004; Valero *et al.*, 2006). Rots in apple are better controlled by carvacrol than by eugenol (Neri *et al*, 2009). It is worth to mention that there is always an important risk of

conferring off-odours when applying essential oils. Sensory evaluation, particularly for FC produces is a point to take seriously into account.

Essential oils from plant also have antioxidant and anti-inflammatory properties for humans. They could be used for extending shelf-life of FC products and, at the same time, to confer other benefits for consumers' health (Gutiérrez *et al.*, 2009).

The use of natural aroma compounds to improve shelf-life and safety of FC fruits has been reviewed (Lanciotti *et al.*, 2004). Plant volatiles have been widely used as food flavouring agents and most of them are generally recognized as safe (GRAS) (Newberne at al., 2000). The addition of hexanal at levels not exceeding 100 ppm in the storage atmosphere of FC apples reduced the growth rate of natural microflora during storage at 4ºC (Lanciotti *et al.*, 1999). Some research has been conducted on the effect of natural competitive microbiota, in most of the cases lactic acid bacteria and/or their derived antibacterial products for FC plant commodities. It has been suggested that they enhance the safety of FC vegetables (Bari *et al.*, 2005) and apples (Leverentz *et al.*, 2006). However this approach has not been yet fully explored.

An interesting option is the use of edible coatings as carriers of antimicrobial agents. In that way, the effect of lemongrass, oregano oil and vanillin incorporated in apple puree-alginate edible coatings, on the shelf-life of FC Fuji apples, has been investigated. Vanillin containing coatings (0.3% w/w) were the most effective in terms of sensory quality after 2 weeks at 4ºC. All antimicrobial coatings significantly inhibited the growth of psychrophilic aerobes, yeasts and molds. At the same time, lemongrass (1.0 and 1.5% w/w) and oregano oil containing coatings (0.5% w/w) exhibited the strongest antimicrobial activity against *L. innocua* (4 log reduction) (Rojas-Graü *et al.*, 2007). Raybaudi-Massilia *et al.* (2008) studied the effect of malic acid and essential oils of cinnamon, palmarosa and lemongrass and their main active compounds as natural antimicrobial substances incorporated into an alginate-based edible coating on the shelf-life and safety of FC Piel de Sapo melon. Palmarosa oil incorporated at 0.3% into the coating appear to be a promising alternative for FC melon, since it had a good acceptation by panellists, maintained the quality parameters, inhibited the native flora growth and reduced *S. enteritidis* population.

5.3. UV-C radiation and intense pulse lights

UV light at sufficiently short wavelength could be used to break down microorganisms in food, air and water purification systems. The use of non-ionizing, germicidal and artificial UV at a wavelength of 190–280 nm (UV-C) could be effective for surface decontamination of FC products. This treatment offers some advantages to the food industry since it does not leave any residue, does not have legal restrictions; it is easy to use and lethal to most types of microorganisms (Bintsis *et al.*, 2000). In addition, UV-C does not require extensive safety equipment to be implemented (Yaun *et al.*, 2004).

The most important effect of UV-C is at the DNA level by inducing the formation of pyrimidine dimmers which alter the DNA helix and blocks microbial cell replication (Kuo *et al.*, 1997; Lucht *et al.*, 1998). Consequently, cells which are unable to repair radiation-damaged DNA die and sub-lethally injured cells are often subject to mutations (Lado and Yousef, 2002).

Strains sensitivity to UV-C changes depending on the microorganism (Gardner and Shama, 2000). For example *Pseudomonas aeruginosa* was more sensitive than *Micrococcus radiodurans* and *Candida albicans* (Abshire and Dunton, 1981). *In vitro* studies performed by Sumner *et al.* (1995) indicated that UV-C was highly effective in destroying *S. typhimurium* while *L. monocytogenes* and *S. enterica,* also under *in vitro* conditions, were inhibited during 13 days at 5ºC at doses of 2.4 kJ m^{-2} (Escalona *et al.*, 2010).

Related to temperature, UV-C seems to be effective in a range from 5 to 37ºC. However, the structure and surface of the vegetable have an influence on the incident irradiation (Bintsis *et al.*, 2000; Lado and Yousef, 2002; Gardner and Shama, 2000). An interesting benefit of UV-C application is that it stimulates plant defence mechanisms increasing resistance to pathogens. But, from the other side, UV-C may modify cell permeability on vegetables increasing electrolytes, amino acids and carbohydrates leakage, which consequently may stimulate bacterial growth (Nigro *et al.*, 1998). Based on Ben-Yehoshua and Mercier (2005), it is very important to find a safe dose able of greatly weaken microorganisms development without damaging the product.

UV-C light has been evaluated as an alternative for surface disinfection of FC fruit and vegetables (Erkan *et al.*, 2001; Marquenie *et al.*, 2002; Allende and Artés, 2003ab; Yaun *et al.*, 2004). *In vivo* studies on zucchini squash slices exposed to UV-C during 10 and 20 min showed that decay and senescence were delayed by affecting microbial growth (Erkan *et al.*, 2001). However, several products (tomato, Lu *et al.*, 1987; Liu *et al.*, 1993; strawberry; Marquenie *et al.*, 2002; carrot, Mercier and Arul, 1993; table grape, Nigro *et al.*, 1998 and sweet-potato, Stevens *et al.*, 1999) showed an increase in the RR of the slices, while the C_2H_4 production and the sensitivity to chilling injury during storage at 5°C were unaffected. Similar results were found for. Related to the stimulation of defence mechanisms beneficial for health, it has been shown that 4 to 14 kJm^{-2} UV-C applied to broccoli heads delayed yellowing and chlorophyll degradation at 20ºC, with an increased total phenols and flavonoids content, along with higher antioxidant capacity and lower RR (Civello *et al.*, 2006). Some negative effects of the use of UV-C has been observed in FC apple stored for 7 days at 5ºC indicated by an increase in surface browning when 5.6, 8.4 and 14.1 kJ m^{-2} doses were used. This process could be related to an increased enzymatic activity caused by membrane breakage with consequent loss of compartmentalization (Gómez *et al.*, 2010).

The natural microflora of FC Red Oak Leaf lettuce up to 10 days at 5ºC was reduced by the use of two sided UV-C radiation (1.18, 2.37 or 7.11 kJm^{-2}) even when

7.11 kJm^{-2} induced tissue softening and browning after 7 days at 5ºC (Allende *et al.*, 2006). The same trend was also observed for one sided UV-C radiation of shredded Red Oak Leaf and Lollo rosso lettuces throughout 10 days at 5ºC where 0.4 to 8.14 kJ m^{-2} decreased psychrotrophic and coliform bacteria, and yeast growth, but only significant reductions were found when the highest level was applied (Allende *et al.*, 2003ab). The same authors reported an increase in the lactic acid bacteria (LAB) which seems to be stimulated by UV-C, probably due to reduced growth of competitive flora.

Sometimes results are not really consistent, like observed by López-Rubira *et al.* (2005). They analyzed the effect of UV-C doses from 0.56 to 13.62 kJm^{-2} on microbial growth in FC pomegranate arils stored up to 15 d at 5ºC. UV-C doses did not affect RR, but some of them reduced mesophilic, psychrotrophic, LAB and *Enterobacteriaceae* counts. Nevertheless, microbial counts were not systematically reduced throughout shelf-life and yeast and moulds were unaffected.

Combination of MAP and UV-C could have a positive effect by reducing psychrotrophic bacteria, coliform, and yeast growth in FC lettuce without adversely affect sensory quality (Allende and Artés, 2003b). Pre-processing application of UV-C may have also an influence on FC produce's behaviour. Robles *et al.*, (2007) indicated that tomatoes pre-treated with 4 KJm^{-2} UV-C followed by storage under 5 kPa O_2 + 1 kPa CO_2 at 12ºC for 21 days showed retarded ripening and kept better firmness and sensory attributes than air storage.

Decay caused by *Botrytis cinerea* of whole bell peppers harvested from integrated pest management production and treated with 2.27 kJm^{-2} UV-C was reduced after 21 days at 5ºC. A residual effect was found since inhibition remained after a further 5 days period at 15ºC. Bell peppers sticks, also from integrated cultivation, showed a high reduction in mesophilic counts when 2.27 kJ m^{-2} UV-C were applied. On the other hand, sticks from organically cultivated bell peppers, which showed the highest *Enterobacteria* growth, only 2.76 kJ m^{-2} reduced these bacteria counts after shelf-life (Artés *et al.*, 2006a).

The effects UV-C doses to reduce pathogen and spoilage bacterial growth in FC baby spinach has also been studied (Escalona *et al.*, 2010). UV-C doses relatively high (12 or 24 kJ m^{-2}) showed an inhibitory effect on initial microbial counts. However, this effect was lost during storage, reaching higher counts on radiated leaves compared to control. Results were confirmed by Artés-Hernández *et al.* (2009) where UV-C light (4.64, 7.94 and 11.35 kJ m^{-2}) reduced the initial mesophilic and psychrophilic counts on the processing day, but no residual inhibitory effect was found for the higher doses after 6-13 days at 5 and 8ºC. It is possible that high doses of UV-C could stimulate microbial growth by a superficial damage caused on spinach leaves.

Recently, the effects of radiation processing on phytochemicals and antioxidants in plant produce have been reviewed (Alothman *et al.*, 2009). It has been reported that

abiotic stresses like UV light may enhance the nutraceutical content of fresh fruit and vegetables. It would affect the secondary metabolism of fresh produce and increase the synthesis of phytochemicals with nutraceutical activity or reduce the biosynthesis of undesirable compounds (Cisneros-Zevallos, 2003). Also, the impact of lighting on shredded cauliflower packaged in different film types and stored at 4°C in darkness and partial or continuous lighting has been evaluated. The light accelerated browning in the cut zones and the development of abnormal colouring in these areas marked the end of shelf-life. Among the sensory attributes studied, colour was the most affected by exposure to light (Sanz-Cervera *et al.*, 2007). UV-C doses at 0.25 and 1.0 kJ m^{-2} increased anthocyanins concentrations in fresh strawberries (Baka *et al.*, 1999). Bell peppers exposed to 7 kJ m^{-2} UV-C light showed lower total phenolic content and higher antioxidant activity (DPPH scavenging activity) with insignificant effect on the carotenoids content (Vicente *et al.*, 2005). Broccoli florets treated with 4-14 kJ m^{-2} UV-C light displayed lower total phenolic and total flavonoid content along with higher antioxidant capacity compared to control (Costa *et al.*, 2006). Application of 2 or 4 kJ m^{-2} on blueberries did not change the total phenolic content while it increased the total anthocyanins content and FRAP values (Perkins-Veazie *et al.*, 2008). Low UV-C doses (1.6 and 2.8 kJ m^{-2}) for keeping overall quality of FC watermelon have been reported. Low UV-C illumination preserved sensory attributes up to 11 days at 5°C, with maintenance of lycopene and ascorbic acid, and increased total antioxidant capacity (Artés-Hernández *et al.*, 2010).

Intense pulses light (IPL) is an emerging nonthermal technology for the rapid inactivation of pathogenic and spoilage microorganisms in foods. It involves the use of intense pulses of short duration (from 85 ns to 0.3 ms) and a broad spectrum (from 0.45 to 15 Hz) to ensure microbial inactivation on the surface of either foods or packaging materials. The limited energy cost, the lack of residual compounds, and its great flexibility are some of the major benefits of this technique (Elmnasser *et al.*, 2007; Gómez-López *et al.* 2005). It seems to induce structural changes of microbial DNA, comparable to the effect caused by continuous UV sources, but others mechanisms could to be involved (Takeshita *et al.*, 2003). ILP effect is dependent on the light absorption by microorganisms. Moreover, certain food components could also absorb the effective wavelengths and decrease their efficiency. ILP has been used to successfully inactivate *E. coli* O157:H7 on alfalfa seeds (Sharma and Demirci, 2003), and *Aspergillus niger* spores on corn meal (Jun *et al.*, 2003). Regarding shelf-life of FC vegetables, Hoornstra *et al.* (2002) showed that a 2 log CFU/g reduction in aerobic counts on selected vegetables can increase their shelf-life about 4 days. Gómez-López *et al.* (2005) reported that rich in carbohydrate foods such as fruit and vegetables seem to be more suitable for decontamination by ILP, although it was found an increase on the RR of treated produces. In FC vegetables such as spinach, celery, green paprika, soybean sprouts, radicchio, carrot, iceberg lettuce, and white cabbage, a PL treatment of 7 J intensity for 45 and 180 s per side provided log reductions in mesophilic aerobic counts between 0.21 and 1.67, and between 0.56 and 2.04, respectively (Gómez-López *et al.*, 2005). ILP did not prolong the sensory shelf-life of FC white cabbage or Iceberg

lettuce, while from the microbial point of view, 1 extra storage day at 7ºC was achieved for lettuce. Additionally ILP seems to be useful for enzymes inactivation. IPL treatments (4.8, 12, 28 J cm^{-2}) extended the microbiological shelf-life of FC mushrooms by 2-3 days compared with untreated samples. However, high pulse light fluencies (25 and 28 J cm^{-2}) dramatically affected the texture of sliced mushrooms and promoted the enzymatic browning by an increase on PPO activity at the highest dose (Oms-Oliu *et al.*, 2010).

5.4. Ozone

Ozone is a gas at room temperature formed by the highly unstable tri-atomic oxygen molecule (O_3) obtained from the addition of an oxygen atom ($O^{\cdot}$) to a molecular diatomic oxygen (O_2) (Horvath *et al.*, 1985). The gas is colorless with a pungent odor readily detectable at concentrations as low as 0.02 to 0.05 ppm (by volume), which is below levels of health concern. It is a powerful oxidant, second only to the hydroxyl free radical, among chemicals typically used in water treatment. Therefore, it is capable of oxidizing many organic and inorganic compounds in water.

The O_3 is commercially generated by passing O_2 through an electrical charge. Thus, molecular O_2 is split into two $O^{\cdot}$ which are highly reactive moieties. When a free $O^{\cdot}$ encounters O_2, it combines to form the O_3 molecule which rapidly degrades back to O_2 with the released $O^{\cdot}$ combining with another $O^{\cdot}$ to form O_2, or combining with other chemical moieties to cause oxidation (Rice *et al.*, 1981). Upon release of $O^{\cdot}$, O_3 acts as a strong oxidizing agent being very effective in destroying microorganisms (Guzel-Seydim *et al.*, 2004a). O_3 was approved for use as a disinfectant or sanitizer in foods and food processing in the United States (FDA, 1997).

Ozone is scarcely soluble in water and, even when O_3 is more soluble than O_2, NaClO is 12 times more soluble than O_3. Concentrations of O_3 used in water treatment are typically below 14 %, which limits the mass transfer driving force of gaseous O_3 into the water. Consequently, typical concentrations of O_3 found during water treatment range from <0.1 to 1 mg/L, although higher concentrations can be attained under optimum conditions (Hoigné and Bader, 1983). Basic chemistry research has shown that O_3 decomposes spontaneously during water treatment by a complex mechanism that involves the generation of hydroxyl free radicals (Glaze, 1987) which are among the most reactive oxidizing agents in water. The decomposition of O_3 is so rapid in the water phase of food that its antimicrobial action may take place mainly at the surface, leaving no residues. The bactericidal effects of O_3 have been shown on a wide variety of Gram+ and Gram- bacteria as well as spores and vegetative cells (Foegeding, 1985). O_3 destroys microorganisms by the progressive oxidation of vital cell components, preventing the microbial growth and extending the shelf-life of many fruit and vegetables, and their industrial use is increasing (Parish *et al.*, 2003). No significant effect of temperature on the efficacy of O_3 has been noted in the range of 10-26ºC (Ölmez and Akbas, 2009).

The main systems for O_3 application include the gaseous phase storage (for cyclic or continuous exposure) or as ozonated dipping (Aguayo *et al.*, 2006). An increase in the shelf-life of apples and oranges treated with 0.4 ppm O_3 was attributed to the oxidation of C_2H_4 in the cold room (Skog and Chu, 2000). Sarig *et al.* (1996) found that O_3 controlled *Rhizopus stolonifer* and induced resveratrol and pterostilbene phytoalexins synthesis in table grapes, the berries being more resistant to subsequent infection. In fact, 8 ppm O_3 increased up to three fold the resveratrol level in Napoleon table grape (Artés-Hernández *et al.*, 2003) and also the continuous applications of low O_3 doses seem to enhance the total polyphenol content during long-term storage of seedless table grapes (Artés-Hernández *et al.*, 2007). The effect of a flow of 4 ± 0.5 ppm O_3 enriched air applied cyclically (30 min every 3 h) on whole and sliced tomatoes prevented fructose, glucose, ascorbic and fumaric acid losses. In addition O_3 kept a good overall quality of tomato slices and reduced 1.1-1.2 log CFU/g for bacterial and 0.5 log CFU/g for fungal counts (Aguayo *et al.*, 2006). The efficacy of gaseous O_3, applied under partial vacuum in a controlled reaction chamber, for the elimination of *Salmonella* inoculated on melon rind has been investigated (Selma *et al.*, 2008). Gaseous O_3 (10,000 ppm for 30 min under vacuum) reduced viable, recoverable *Salmonella* from inoculated melons with a reduction between 4.2-2.8 log CFU/rind-disk (12 cm^2). A novel ozone-generation system capable of generating O_3 inside a sealed package at various geometries has been developed. Whole prepackaged spinach leaves inoculated with *E. coli* O157:H7 under this ozone-generation system showed that this treatment reduced *E. coli* O157:H7; however, minimizing quality changes after treatment requires further research (Klockow and Keener, 2009).

Conventional sanitizers could be replaced by the use of O_3 water due to its efficacy at low concentrations and short contact time requirements as well as the breakdown to non-toxic products (Graham, 1997). O_3 solubility has a strong influence on the efficacy of this treatment. Solubility depends on temperature, increasing with decreased water temperature (Bablon *et al.*, 1991). It is important to provide certain turbulence to the water as a way of increasing bubble contact and subsequent O_3 solubilisation. The flow rate and contact time as well as purity and pH of water are also important and greatly affect the rate of O_3 solubilisation. Kim *et al.* (1999a) indicated that high pH destabilizes O_3 and decreases the apparent rate of solubilisation. Moreover, tap water may contain organic matter that consumes O_3.

Inoculating targeted microorganisms on pure cell suspensions or on the food surface and treating these surfaces with O_3 under conditions that simulate normal processing has been useful for studying the efficacy of O_3 water. In that way some authors (Restaino *et al.*, 1995; Singh *et al.*, 2002) have found a decrease in pathogens including *S. aureus, S. typhimurium, Y. enterocolitica, L. monocytogenes* and *E. coli* O157:H7. The use of 1.5 ppm O_3 water (pH = 6, 25ºC) for 15 s reduced between 1.5 and 5 log CFU/g *E. coli* O157:H7, *P. fluorescens*, *L. mesenteroides* and *L. monocytogenes* counts (Kim *et al.*,1999b). Khadre and Yousef (2001) indicated that effectiveness of O_3

in disinfecting food-contact surfaces may be tested using spores of *B. stearothermophilus* as indicators, due to their high resistance to O_3.

Food composition may have a significant effect on the bactericidal power of O_3 against spore former, a Gram$^-$ rod, and a Gram$^-$ cocci. High levels of protein (caseinate) and fat (whipping cream) resulted in the greatest amount of protection to spores of *B. stearothermophilus* and vegetative cells of *E. coli* and *S. aureus* (Guzel-Seydim *et al.*, 2004b). O_3 may have a positive influence on some unwanted changes like Zhang *et al.* (2005) reported for celery sticks. In this case, dipping 0.18 ppm O_3 water for 5 min reduced RR, inhibited browning and improved sensory quality. After 9 d at 4ºC, bacterial population was reduced 1.69 log CFU/g compared to control water. Rico *et al.* (2006) found that O_3 water (1 mg L^{-1} at 18–20ºC) reduced both enzyme activity and enzymatic browning of shredded lettuce. However, this enzyme inactivation showed a negative effect, as the reduction in activity of the texture-related pectin methylesterase was correlated with a lower crispiness. Selma *et al.* (2007) treated shredded lettuce with 5 ppm O_3 water for 5 min reaching a reduction of 1.8 log CFU/g in *Shigella sonnei* counts. Silveira *et al.* (2007; 2010) found a similar sanitizer effect between 150 ppm NaClO and O_3 dips (0.4 ppm, 3 min) on diced Galia melon. Both treatments reduced 1 log CFU/g compared to water alone and extended shelf-life to 10 days at 5ºC.

In some cases O_3 appears to be lowly successful. Aguayo *et al.* (2003) washed 3 min FC Amarillo melon on 6.5 ppm O_3 water. After 10 days at 5ºC, the mesophilic and psychrotrophic bacterial counts were reduced only on 1.2 and 0.2 log CFU/g respectively and on 0.4 and 1.5 log CFU/g respectively those of yeasts and moulds. In sliced Thomas tomato stored for 10 days at 5ºC, the O_3 water (3.8 ppm, 3 min) reduced on 1.9, 1.6 and 0.7 the mesophilic, psychrotrophic and yeast counts respectively. In shredded Iceberg lettuce, Baur *et al.* (2005) maintained better sensory and microbiological quality, prolonging shelf-life, in samples treated with 200 mg/L free chlorine than in O_3 water (1 ppm, 4ºC, 120 s). In contrast, the application of 2 ppm O_3 water for 2 min was found to be optimum processing conditions of shredded green leaf lettuce, in terms of reducing microbial load and maintaining the sensory quality compared with 100 ppm NaClO water during chilling storage (Ölmez and Akbas, 2009).

Beltrán *et al.* (2005) found no evidence of browning in FC potatoes dipped in O_3 water (20 mg L^{-1} 1 min) or in O_3 plus peroxyacetic acid (300 mg L^{-1}) and stored up to 14 d under partial vacuum at 4ºC. These treatments kept the initial texture and aroma. However, the ozonated water alone was not effective in reducing total microbial populations. The combination of O_3–Tsunami 100 (15% PA and 11% H_2O_2) was the most effective treatment to control microbial growth achieving 3.3, 3.0 and 1.2 log-reductions for LAB, coliforms and anaerobic bacteria, respectively.

Regarding O_3 handling, even when it has been approved as food additive, workers who might have contact with O_3 must be careful since gas concentrations over

0.2 ppm may affect respiration and may produce dizziness and eyes and throat irritation (Hoof, 1982). Ambient O_3 levels at the industry facilities should be continuously monitored in view of the fact that high levels may be fatal to humans (Shirk, 2000)

5.5. Superatmospheric oxygen

For most of the cases, a conventional MAP with 1 to 8 kPa O_2 combined with 10 to 20 kPa CO_2 at 0 to 5°C is frequently recommended for keeping quality of FC fruit and vegetables (Gorny, 2003; Artés *et al.*, 2006b). However, the combined effect of high O_2 concentrations (over 60 kPa) and CO_2 around 10 to 20 kPa may provide adequate suppression of microbial growth increasing produce shelf-life (Allende *et al.*, 2004; Geysen *et al.*, 2006; Conesa *et al.*, 2007ab; Escalona *et al.*, 2007). This superatmospheric O_2 MAP could be effective for inhibiting enzymatic browning, preventing anaerobic fermentation and moisture and odour losses while reducing aerobic and anaerobic microbial growth (Day, 2001).

A number of factors may explain the toxicity of hyperbaric O_2 like the adverse effects on the oxidation-reduction potential of the system, the oxidation of enzymes having sulfhydryl groups or disulfide bridges, and the accumulation of injurious reactive O_2 species (Kader and Ben-Yehoshua, 2000). CO_2 causes a decrease in intra and extracellular pH and interferes with the cellular metabolism with a stronger inhibitory effect at low temperature due to enhanced CO_2 solubility (Dixon and Kell, 1989).

Recently, some studies have been carried out for analysing the effect of high O_2 and CO_2 levels on quality and safety of FC plant commodities (Allende *et al.*, 2004; Amanatidou *et al.* 1999, 2000; Geysen *et al.*, 2006; Conesa *et al.*, 2007a,b; Escalona *et al.*, 2007; Jacxsens *et al.* 2001; Sánchez-Ballesta *et al.*, 2007). In practice, the use of high O_2 in MAP for keeping quality of FC produces is still emergent and needs to be supported by more research. Kader and Ben-Yehoshua (2000) have found some contradictory results since superatmospheric O_2 may stimulate, have no effect, or reduce RR and C_2H_4 production, depending on the commodity, ripening stage, O_2, CO_2 and C_2H_4 concentrations in the atmosphere, storage time and temperature. Related to microbial growth, it seems to be that exposure to high O_2 did not strongly inhibited it, while high CO_2 reduced the growth to some extent in most cases. Allende *et al.* (2004) found a reduction of aerobic microbial growth in superatmospheric O_2 due to the accumulation of high CO_2 levels. The antimicrobial activity of CO_2 at high concentrations is already well known. Probably the same effect was observed by Van Der Steen *et al.* (2002) for raspberries and strawberries when applying a high O_2 level in a high-barrier film, showing a reduction in microbial growth while keeping sensory quality attributes. The addition of CO_2 is unnecessary when high O_2 atmospheres are injected in barrier film packages, because the respiratory activity of produces generate antimicrobial CO_2 levels reaching 20 kPa. By using this high O_2 MAP the shelf-life for FC vegetables can be twice compared to conventional low O_2 MAP just by suppressing

microbial growth (Jacxsens *et al.*, 2001). In the case of *L. innocua* levels higher than 75 kPa O_2 were needed to reduce growth on shredded lettuce (Escalona *et al.*, 2007), in agreement with other studies where *L. innocua* growth was almost not influenced by O_2 concentrations up to 100 kPa (Geysen *et al.*, 2006). *In vitro* studies on yeast growth under superatmospheric O_2 showed contradictory results. Jacxsens *et al.* (2001), found a reduced growth of *Candida lambica* while a stimulated growth of *C. guilliermondii* and *C. sake* under around 80 kPa O_2 at 8°C was reported by Amanatidou *et al.* (1999). Yeast growth can be reduced or stimulated under high O_2 levels depending on the species or strain and their sensitivity to high O_2 (Van der Steen *et al.*, 2003). Allende *et al.* (2002), reported yeast counts higher than 5 log CFU/g after 3 days at 4°C on mixed salad stored under superatmospheric O_2 MAP. For FC melon, high O_2 levels kept the initial colour and firmness retarding anaerobic fermentation better than low O_2 concentrations. In addition, yeast growth was delayed (Oms-Oliu *et al.*, 2008b). Related to residual effect Zheng *et al.* (2008), found that berries kept in 60 to 100 kPa O_2 showed inhibited decay and, after additional 2 days in air at 20°C, treated fruit also exhibited less decay rate, suggesting that high O_2 levels had residual effect on decay control.

Linked to the response of FC vegetables to high O_2, Jacxsens *et al.* (2001) reported a low sensitivity in grated celeriac and shredded chicory endive. In 3, 80 and 95 kPa O_2, grated celeriac reached 120, 97 and 124 nmol O_2 /kg s respectively and for shredded endive the RR was 90, 107 and 132 nmol O_2 /kg s also respectively. In addition, exposure of shredded carrot to 50 kPa O_2 + 30 kPa CO_2 showed an accelerated C_2H_4 production, possibly as an injury response at 8°C after 2 to 3 days (Amanatidou *et al.*, 2000). Escalona *et al.* (2006) reported a higher effect of CO_2 than that of O_2 in the RR of FC butter lettuce. A level of 10 kPa CO_2 reduced the RR and when raised over 20 kPa CO_2 the RR increased probably due to a shift to anaerobic metabolism. At 20 to 100 kPa O_2 and 1, 5 and 9°C, the respiratory quotient was about 0.7 to 1. In FC carrot stored in initial 70 kPa O_2, the RR expressed in O_2 consumed was 3-fold higher than under initial 2.5 kPa O_2 + 7 kPa CO_2 as a consequence of oxidative processes. By using low permeability film a rapid depletion of O_2 and accumulation of CO_2 was obtained under initial low O_2 concentration (Oms-Oliu *et al.*, 2008a).

Few reports indicate the effects of elevated O_2 on enzymatic browning. From *in vitro* studies of PPO kinetics, the main enzyme responsible of browning, it seems to be that the substrate concentration as well as the O_2 level had a clear inhibitory effect on the reaction rate. Moreover, the inhibitory effect of O_2 was more evident at low final product concentration (Gómez *et al.*, 2006). *In vivo* studies showed that an atmosphere of 80 kPa O_2 + 20 kPa CO_2 delayed browning of shredded lettuce under MAP for 10 days at 5°C compared to air (Heimdal *et al.*, 1995), although it is well know that browning susceptibility is highly cultivar dependent. Jacxsens *et al.* (2001) found that low O_2 caused a reduction in the enzymatic browning in FC vegetables since O_2 is a necessary substrate for the reaction. However, high O_2 atmospheres were more effective in reducing or inhibiting the enzymatic browning of Iceberg lettuce, radicchio and Lollo Rosso lettuce. In the case of FC bell pepper, 80 kPa O_2 combined with 15 kPa CO_2

maintained the main sensory quality attributes and inhibited growth of the spoilage microorganisms and *Enterobacteriaceae* (Conesa *et al.*, 2007ab).

In the case of shredded Iceberg lettuce Heimdal *et al.* (1995) found that under 80 kPa O_2 + 20 kPa CO_2 browning was higher than under moderate vacuum MAP. For Spartan apple, Lu and Toivonen (2000) reported that a pretreatment with 100 kPa O_2 before cutting reduced surface browning, flesh softening, and off-flavour in slices. This inhibition of browning was associated with retention of cellular integrity.

Regarding sensory quality, acceptable scores were found in FC spinach leaves treated with 80 to 100 kPa O_2 + 20 kPa CO_2 compared to low O_2 and high CO_2, where the spinach was affected by fermentation (Allende *et al.*, 2004). Beneficial effects of superatmospheric O_2 have been reported for shredded chicory endive and mixed vegetable salads (Jacxsens *et al.* 2001; Allende *et al.*, 2002; Escalona *et al.*, 2007).

In addition, FC carrots stored in 50 kPa O_2 + 30 kPa CO_2 had similar or better quality than those in 1 kPa O_2 + 10 kPa CO_2 after 12 days at 8°C. Therefore, FC carrots could tolerate high CO_2 levels in combination with high O_2 levels, and this innovative MAP can be used for keeping their fresh characteristics and lowering microbial growth during shelf-life (Amanatidou *et al.*, 2000). FC butter lettuce kept a good visual appearance with 75 kPa O_2 + 15 kPa CO_2 after 10 days at 7°C, reducing cut surface browning and loss of freshness (Escalona *et al.*, 2007). Spinach leaves kept a better appearance when stored at higher than 50 kPa O_2, delaying anaerobic fermentation and increasing shelf-life when compared to atmospheres with 0.5 and 25 kPa O_2 (Magalhães *et al.*, 2007).

5.6. Innovative gas treatments

Packaging under non-conventional gases like Ar, He, Xe or N_2O has been proposed for improving quality of some FC plant commodities. Those gases may be chemically inert, but they had some antimicrobial and/or physiological effects, although it did not appear to be through modification of enzymes activity (Gorny and Agar, 1998). In fact, it has been shown that an Ar-enriched MAP reduced microbial growth and delayed quality loss of fresh broccoli and lettuce (Day, 1996; Jamie and Saltveit, 2002). In that way, a patent registered the use of Ar in the preservation of cut and segmented fresh fruits (Powrie *et al.*, 1990). He or Ar-enriched atmospheres might modify the O_2, CO_2, and C_2H_4 diffusion in some commodities (Burg and Burg, 1965). Certainly, low O_2 atmospheres combined with high Ar, He or N_2 had different diffusive characteristics because both Ar and He are monatomic gases and are smaller in size than the diatomic N_2 (Jamie and Saltveit, 2002). It has been found that 90 kPa Ar + 2 kPa O_2 did not delay the accumulation of phenolics in FC lettuce, or the loss of chlorophyll from broccoli florets beyond that of low O_2 atmospheres made with He or N_2 (Lougheed and Lee, 1991). According to Sidorkin *et al.* (1989) there was no difference in the viability of cells, the uptake of sucrose, or the density of tobacco suspension cultures

grown for 8 days in 21 kPa O_2 and 79 kPa of Ar or N_2. On the other hand Xe showed good results on forming clathrate hydrates (crystalline water-based solids) under 1.5 MPa pressure in preservation of asparagus and cucumber, but the high cost may limit its application. Storing asparagus spears at 4 ºC under 1.1 MPa mixture of Ar and Xe (2:9, v:v) for 24 h extended their shelf-life compared with a conventional MAP of 5 kPa O_2 + 5 kPa CO_2 (Zang *et al.*, 2007).

N_2O has effects as an inhibitor of C_2H_4 production and RR on fruits (Benkeblia & Varaquaux, 2003; Palomer *et al.*, 2005) as well as a fungistatic agent (Thom and Marquis, 1984; Qadir & Hashinaga, 2001a,b). N_2O has 77% solubility in fruit cell, although its absorption in tissues is completely reversible (Gouble *et al.*, 1995). Therefore, N_2O have a direct effect by extending the shelf-life of these products. Innovative MAP of 90 kPa Ar + 5 kPa O_2 and 90 kPa N_2O + 5 kPa CO_2 were used with kiwifruit slices at 4 ºC for 12 days in comparison to air and N_2-enriched storage. MAP with 90 kPa N_2O maintained the best quality of slices, delaying firmness loss and browning in pericarp and core surfaces. Slight modifications in the most important discriminated quality factors for the slices under N_2O-enriched, a rapid quality loss for air and N_2-enriched atmosphere and an acceptable quality in Ar-enriched atmospheres after 8 days at 4ºC were found (Rocculi *et al.*, 2005). FC spinach leaves fertilized with 8 or 16 mmol N /L under a floating trays system and stored under N_2O-enriched MAP showed after 8 days at 5ºC a reduced microbial growth, with good sensory quality preserving the total antioxidant capacity, phenolics and chlorophylls content (Rodríguez-Hidalgo *et al.*, 2010).

5.7. Conclusions and future trends

The food industry is now seeking alternatives to chlorine which may assure the safety of FC produce and maintain the quality and shelf-life, while also reducing the rate of water consumption during processing. Sustainable strategies that would allow replacing it are promissory. Chlorine dioxide, ozone, organic acids, peroxyacetic acid, electrolyzed oxidizing water, UV-C and hydrogen peroxide are the main alternative sanitizing agents that gained interest in recent years. The effects of these disinfecting agents on the microbiological, nutritional and sensory quality of FC produce, and also the possible environmental impact and the potential on minimizing water consumption rates in the food industry are aspects to be elucidated during the next years. The potential and limits of these innovative sustainable techniques must also be well defined and included in the regulations.

It is worth to mention that genetic *cv* selection must be oriented to slow ripening and senescence rates, with low C_2H_4 production and/or sensitivity, improved firmness, good adaptation to minimal processing and enhanced antioxidant systems.

Application of a sanitizer or a combination of disinfection treatments does not mean a sanitation program. It implies much more effort, from a well designed integrated

production, handling and processing to proper distribution chains, respecting low storage temperatures and optimal MAP. In that way, food safety hazards associated with FC produce must be clearly identified. It should be taken into account that the produce moves from the field through the processing plant and then to the consumer's table. Consequently three highly important sources of contamination -microbial, chemical and physical - may represent a health hazard. Procedures like HACCP together with a specific action plan should be developed on each case in order to identify and control any of these hazards from entering the handling process.

How to interpret microbial results and which standardized procedures are best for consistent microbial analysis in testing FC produce must be a top priority. Consequently, new techniques for microbial detection like PCR and qPCR have to be promoted and fitted for different crops and handling conditions.

Table 5.1. Alternative sanitizers to chlorine for fresh-cut fruit and vegetables.

SANITIZER	COMMODITY	REFERENCES
Peroxiacetic acid	Melon	Rodgers *et al.*, 2004; Rocha-Bastos *et al.*, 2005; Silveira *et al.*, 2010
	Carrot, lettuce, leek, white cabbage	Ruiz-Cruz *et al.*, 2007; Vandekinderen *et al.*, 2009
	Tomato, sweet pepper, cucumber	Alvaro *et al.*, 2009
	Rocket	Martínez-Sánchez *et al.*, 2006
	Fresh-cut potato	Beltrán *et al.*, 2005
	Escarole, lettuce	Rodgers *et al.*, 2004; Allende *et al.*, 2008; López-Gálvez *et al.*, 2009
	Apples, strawberries	Rodgers *et al.*, 2004
Chlorine dioxide	Cabbage	Sy *et al.*, 2005; Gómez-López *et al.*, 2008
	Lettuce	Rodgers *et al.*, 2004; Sy *et al.*, 2005, Gómez-López *et al.*, 2008; Mahmoud and Linton, 2008a,b; López-Gálvez *et al.*, 2010
	Carrot	Sy *et al.*, 2005; Gómez-López *et al.*, 2007
	Strawberry	Rodgers *et al.*, 2004; Mahmoud *et al.*, 2007
	Melon	Sy *et al.*, 2005; Rodgers *et al.*, 2004
	Apple	Rodgers *et al.*, 2004
	Faba bean	Artés *et al.*, 2007a,b
	Chard	Tomás-Callejas *et al.*, 2010
	Green pepper	Han *et al.*, 2001
Hydrogen peroxide	Melon	Sapers and Simons, 1998; Ukuku *et al.*, 2004, 2005
	Lettuce	Gopal *et al.*, 2010
	Green bell pepper, cucumber, zuchini	Sapers, 2003
Citric acid – ascorbic acid – calcium	Chinese cabagge	Kim and Klieber, 1997
	Melon	Luna-Guzmán *et al.*, 1999; Aguayo *et al.*, 2003, 2007

	Lettuce	Martín-Diana *et al.*, 2006a; Ölmez and Temur, 2010
	Green celery	Gómez and Artés, 2004
	Cilantro	Allende et al., 2009
Electrolyzed water	Carrot	Izumi, 1999
	Spinach	Izumi, 1999
	Bell pepper	Izumi, 1999
	Potato	Izumi, 1999
	Cucumber	Izumi, 1999
	Lettuce	Hua *et al.*, 2004; Koseki *et al.*, 2001; Abadías *et al.*, 2008; Rico *et al.*, 2007a
	Alfalfa sprouts	Kim *et al.*, 2003
	Cilantro	Wang *et al.*, 2004
	Mizuna leaves	Martínez-Hernández *et al.*, 2009
Steamer jet injection	Lettuce	Martín-Diana *et al.*, 2007
Biological compounds, natural microbiota and/or their antimicrobial compounds	Lettuce	López-Gálvez *et al.*, 2009; Xu *et al.*, 2007; Martín-Diana *et al.*, 2008; Gutierrez *et al.*, 2009
	Cucumber	Xu *et al.*, 2007
	Apple	Lanciotti *et al.*, 1999; Leverentz *et al.*, 2006
	Carrot	Gutierrez *et al.*, 2009
	Cabbage	Bari *et al.*, 2005
	Broccoli	Bari *et al.*, 2005
	Mung bean sprouts	Bari *et al.*, 2005
UV-C radiation and intense light pulses	Spinach	Artés-Hernández *et al.*, 2008; Escalona *et al.*, 2010
	Zucchini squash	Erkan *et al.*, 2001
	Tomato	Lu *et al.*, 1987; Liu *et al.*, 1993; Robles *et al.*, 2007
	Strawberry	Marquenie *et al.*, 2002; Allende *et al.*, 2007
	Lettuce	Allende *et al.*, 2003a,b, 2006; Gómez-López *et al.*, 2005
	Pomegranate arils	López-Rubira *et al.*, 2005
	Bell pepper	Artés et al., 2006
	Mushroom	Oms-Oliu *et al.*, 2010
	Watermelon	Fonseca and Rushing, 2006; Artés-Hernández *et al.*, 2010
Ozone	Lettuce	Baur *et al.*, 2005; Rico *et al.*, 2006; Selma *et al.*, 2007; Ölmez and Akbas, 2009
	Apple	Skog and Chu, 2000
	Orange	Skog and Chu, 2000
	Table grape	Sarig et al., 1996
	Tomato	Aguayo *et al.*, 2006

	Melon	Aguayo *et al.*, 2003; Silveira *et al*, 2007, 2010; Selma *et al.*, 2008
	Spinach	Klockow and Keener, 2009
	Celery	Zhang *et al.*, 2005
	Potato	Beltrán *et al.*, 2005
Superatmospheric oxygen	Mixed salads	Allende *et al.*, 2002, 2003; Amanatidou *et al.*, 1999, 2000
	Carrots	Oms-Oliu *et al.*, 2008a
	Bell pepper	Conesa *et al.*, 2006, 2007
	Table grapes	Sanchez-Ballesta *et al.*, 2001
	Strawberry	Van Der Steen *et al.*, 2002; Zheng *et al.*, 2008
	Melon	Oms-Oliu *et al.*, 2008b
Innovative gas treatments	Asparagus	Zang *et al.*, 2007
	Apple	Qadir and Hashinaga, 2001
	Guava	Qadir and Hashinaga, 2001
	Mandarin	Qadir and Hashinaga, 2001
	Persimmon	Qadir and Hashinaga, 2001
	Strawberry	Qadir and Hashinaga, 2001
	Tomato	Qadir and Hashinaga, 2001

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OBJECTIVES

OBJECTIVES

The current research work is focused on keeping quality and food safety during postharvest handling, processing and shelf life of fresh-cut leafy greens and whole tomato. Chlorination with NaClO, as an industrial disinfection technique for plant commodities, has been forbidden in some European countries and this may lead to new regulatory restrictions in the near future. Different issues such as dangerous by-products formation, poor efficiency in some commodities and gas emission, which can affect workers and consumers safety, are related to its use.

In this Thesis, an integrated perspective of food preservation where strategies to keep or enhance quality and sensory attributes of vegetables are combined with strategies to maintain safety has been the major approach to address, develop and solve the raised problems. The research work focuses on the evaluation of new emerging alternatives to replace chlorine in the fresh and fresh-cut produce industry. These integrated preservation techniques include the use and optimization of alternative chemical sanitizers, physical pre-packing treatments and design of an optimal passive or active modified atmosphere packaging.

The specific objectives of this Thesis are:

- In baby leafy vegetables:
    - Evaluation and optimization of alternative chemical sanitizers (ClO_2, acidified sodium chlorite, electrolyzed water) to chlorine and its effects on microbial, nutritional and sensory quality during shelf life.
    - Evaluation throughout shelf life of innovative active MAP treatments (enriched atmosphere in noble gases, superatmospheric oxygen and nitrous oxide) on the overall produce quality.
    - Evaluation of the use of a UV-C radiation pre-treatment and in combination with passive and active MAPs in quality changes throughout shelf life.
    - Potential risk assessment of cross contamination by *Salmonella enterica* and *Escherichia coli* O157:H7 during fresh-cut processing by using chemical sanitizers.
    - Study of the survival and distribution of *E. coli* from production to retail distribution.
- In tomato:
    - Development of scientifically-based critical operating standards for ClO_2 in postharvest washing of fresh tomatoes.
    - Evaluation of the influence of water quality in ClO2 effectiveness to inactivate *S. enterica.*

CHAPTER 1

Innovative active modified atmosphere packaging improves overall quality of fresh-cut Red Chard baby leaves

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Innovative active modified atmosphere packaging improves overall quality of fresh-cut red chard baby leaves

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ABSTRACT

The antimicrobial effects and quality changes of O_2, He, N_2 or N_2O-enriched active modified atmosphere packaging –MAP– (100 kPa initial gas partial pressure) compared to a chlorinated (100 mg L^{-1} NaClO–passive MAP (control) in fresh-cut Red Chard baby leaves up to 8 days at 5 °C were studied. High O_2 MAP (>85 kPa O_2) inhibited natural microflora growth during 7 days at 5 °C. Regarding control treatment, no differences for He and N_2O-enriched MAPs on microbial growth were found although control samples were previously disinfected. Initial total phenolics content (613 mg ChAE kg^{-1} fw) increased up to 61–93% after 6 days at 5 °C under O_2, He, and N_2-enriched MAPs. Vitamin C content decreased up to 87% after shelf life in control samples while lower decreases were monitored in samples stored under non conventional MAPs. He-enriched MAP preserved the total chlorophylls content throughout shelf life. After 8 days at 5 °C the overall sensory quality of all treatments showed a moderate decrease while still being scored at the limit of usability. In conclusion, He and O_2 enriched MAPs are useful tools in the preservation of fresh-cut Red Chard quality.

1. Introduction

The current lifestyle with little time to prepare a convenient meal and to have a balanced diet has created a new kind of consumer who demands fresh, healthy and ready-to-eat products. Fresh-cut vegetables are products which fit within the new consumer trends. Several studies have reported the nutritional contents of these fresh leaves. Pyo, Leeb, Logendrac, and Rosenb (2004) reported the antioxidant activity and phenolic compounds of Swiss Chard extracts as a good dietary source of phenolic antioxidants. In addition, Chard is rich in vitamin C (Gil, Ferreres, & Tomás-Barberán, 1998). Non-conventional active MAP is a novel technique for packaging plant commodities which replace the original atmospheric gas partial pressure with noble gases (He, Ar or Xe), nitrous oxide (N_2O), N_2, or superatmospheric O_2 concentrations that might be favourable for keeping its quality (Artés, Gómez, Aguayo, Escalona, & Artés-Hernández, 2009). The effects of superatmospheric O_2 MAP on quality changes in several leafy vegetables have been reported (Allende, Aguayo, & Artés, 2004; Artés & Allende, 2005), although it needs to be supported by more research. Little information about the effects of non-conventional MAP on fresh-cut baby leaves on overall and microbial quality is available and not completely clear. Zhang, Zhan, Wang, and Tang (2007) have shown that storing asparagus spears with 1.1 MPa mixture of Ar and Xe (2:8, v:v) for 24 h at 4 °C extended their shelf life compared to a conventional MAP. In addition, the effects of N_2O as an inhibitor of C_2H_4 production and RR (Palomer, Roig-Villanova, Grima-Calvo, & Vendrell, 2005) as well as on fungistatic effect on fruits have been demonstrated (Qadir & Hashinaga, 2001; Rodríguez–Hidalgo, Artés–Hernández, Gómez, Fernández & Artés, 2010). The objective of the present work was to evaluate the effect of four non-conventional active MAPs (100 kPa O_2, 100 kPa He, 100 kPa N_2 and 100 kPa N_2O of initial gas partial pressures) compared to a conventional passive MAP on overall quality changes of fresh-cut Red Chard baby leaves during shelf life. To the best of our knowledge, this is the first report to study the effects in quality changes of He and N_2O-enriched atmospheres of fresh-cut Red Chard.

2. Materials and methods

2.1. Plant material

Baby Red Chard (*Beta vulgaris* var. cycla) leaves were grown in Mediterranean climate (Murcia, Spain) and mechanically harvested at a commercial development stage. Immediately after harvesting, the leaves were transported to the Technical University of Cartagena where they were pre-cooled by air at 5 °C until further processing.

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2.2. Sample preparation, treatments and storage conditions

Baby leaves were hand processed in a clean room at 8 °C and those with defects were discarded. Red Chard leaves were prewashed for 1 min with tap water at 5 °C for active non-conventional MAP treatments, the leaves were washed in tap water for 2 min at 5 °C, while for control treatment they were washed with chlorinated water (100 mg L^{-1} NaClO) at 5 °C for 2 min. After washing, leaves were rinsed by immersion in tap water at 5 °C for 1 min, and then were spin dried to eliminate excess water. About 40 g of leaves were placed in polypropylene (PP) trays of 1500 mL capacity and thermally sealed with a bi-oriented PP (BOPP) (Plásticos del Segura S.L., Murcia, Spain). For control, a 40 μm thickness BOPP to generate a passive MAP was used. O_2TR and CO_2TR at 23 °C and 0% RH was similar with 800 cm^3 m^{-2} d^{-1} atm^{-1} and WVTR at 23 °C and 85% RH was 0.7 g m^{-2} d^{-1} atm^{-1} (data provided by the supplier). Four non-conventional active MAP treatments initially composed of 100 kPa of O_2, He, N_2 and N_2O were assayed. In all cases, the trays were thermally sealed at the top with a 50 μm thickness BOPP just after flushing the corresponding gas within packages. O_2TR and CO_2TR at 23 °C and 0% RH was similar with 500 cm^3 m^{-2} d^{-1} atm^{-1} and WVTR at 23 °C and 85% RH was 0.5 g m^{-2} d^{-1} atm^{-1} (data provided by the supplier). Five replicates of one basket per treatment and storage time (processing day and after 4, 6 and 8 days) were prepared and stored at 5 °C.

2.3. Analysis and determinations

2.3.1. Respiration rate and gas composition within packages

The RR of the leaves was determined at 5 °C by using a closed system. Three replicates of 20 g leaves were placed within 750 mL glass jars at 5 °C up to 8 days. The increases in CO_2 were monitored almost daily starting at 2.5 h after having closed the jars. Headspace gas samples (1 mL) were withdrawn from the jars with a gas-tight syringe and analyzed in a gas chromatograph (GC) (Thermo Finnigan Trace CC, Milan, Italy), equipped with a thermal conductivity detector (150 °C), oven (ramp from 40 to 90 °C), injector (150 °C) and Porapack-N 80/100 column. He (20 mL min^{-1}) was the carrier gas. In order to avoid CO_2 accumulation and to maintain a high RH within jars, a continuous flow of 30 mL min^{-1} of humidified air was applied between each observation. Gas composition (O_2, CO_2 and N_2) within packages was monitored on processing day and after 4, 6 and 8 days of storage. Headspace gas samples (1 mL) were withdrawn from the packages using a gas-tight syringe and analyzed in the GC. He and N_2O partial pressures were calculated as remaining partial pressure after adding O_2, CO_2 and N_2. Three replicates were made for each treatment and evaluation period.

2.3.2. Colour

Leaf colour was measured at three points on the upper side of leaves using a compact tristimulus colorimeter (Minolta CR-300, Ramsey, NJ, USA) with an 8 mm diameter viewing aperture and a white plate C reference ($Y = 94.3$, $x = 0.3142$, $y = 0.3211$, standard CIE illuminant, 2° observer). Values were expressed as Hunter lightness, chroma and hue angle parameters. Observations were made on thirty randomly selected leaves. All treatments were replicated three times.

2.3.3. Microbial analysis

Standard enumeration methods were used to determine the microbial growth. Three random samples were taken on each evaluation time. Ten grams of leaves were homogenized in 90 mL of sterile buffered peptone water (BPW) (Scharlau Chemie SA, Barcelona, Spain) for 1 min in a sterile stomacher bag (Model 400 Bags 6141, London, UK) using a Masticator (Colwort Stomacher 400 Lab, Seward Medical, London, UK). In order to determine the enumeration of each microbial group (mesophilic, enterobacteria, psychrotrophic, yeasts and moulds), ten-fold dilution series were prepared in 9 mL of BPW. The following media and incubation conditions were used: plate count modified agar (Scharlau Chemie, Barcelona, Spain) for mesophilic and psychrotrophic aerobic bacteria, incubated at 30 °C for 48 h and at 5 °C for 7 days respectively; violet red bile dextrose agar (VRBD) (Scharlau Chemie, Barcelona, Spain) for enterobacteria, incubated at 37 °C for 48 h; and potato dextrose agar base (Scharlau Chemie, Barcelona, Spain) with oxytetracycline (100 mg L^{-1}) (Sigma Chemical Co., St Louis, MO, USA) for yeasts and moulds, incubated for 3–5 days at 22 °C. All microbial counts were reported as log colony forming units per gram (log CFU g^{-1}). The presence of *Salmonella* spp., *Listeria monocytogenes* and generic *Escherichia coli* was also evaluated according to the EU legislation for fresh-cut vegetables (Regulation EC 1441/2007, 2007). All analyses were made in triplicates.

2.3.4. Sensory evaluation

A seven person panel (aged 24–62) trained in sensory quality analyses performed the evaluation. Before running the experiments a consensus was reached between the panellists in order to select those attributes that better describe sensory changes. Sensory evaluation was evaluated on processing day and after 4, 6 and 8 days at 5 °C. Dehydration, browning, off-odours and off-flavours were scored on a five-point scale of damage incidence and severity (1 = none, 2 = slight, 3 = moderate, 4 = severe and 5 = extreme). Visual appearance and overall quality was evaluated by using a nine-point scale (1 = extremely poor, 5 = fair, limit of usability, 9 = excellent).

2.3.5. Chlorophyll content

For tissue preparation, 25 g of leaves from each treatment were frozen in liquid N_2, grinded using a mincer (IKA, A 11 basic, Berlin, Germany) at 28,000×*g* for 10 *s*, and stored at −80 °C. The sample preparation for chlorophyll determination was conducted according to Smith & Benitez (1955). The total chlorophyll pigments were extracted with: hexane. A 0.5 g sample of frozen shredded leaves was mixed with 9 mL of hexane and 15 mL of a mixture of methanol:acetone (1:2). The extraction was carried out for 5 h at 5 °C in darkness. Then the samples were shaken at 200×*g* every 15 min by using a vortex (Velp Scientifica, Zx^3, Milano, Italy). After incubation, 25 mL of 1 M NaCl was added. Subsequently the samples were shaken again and then centrifuged for 30 min at 2600×*g* at 4 °C. After centrifugation, 1 mL of the supernatant was pipetted into a quartz cuvette (Hellma GmbH & Co., Müllheim, Germany). The equations developed by Wellburn (1994) were used to determine the individual levels of both chlorophyll *a* (Chl *a*) = $10.05\,A_{662} - 0.766\,A_{644}$ and chlorophyll *b* (Chl *b*) = $16.37\,A_{644} - 3.14\,A_{662}$ where total chlorophylls amount was calculated as Chl *a* + Chl *b*. The absorbance (*A*) at 662 and 644 nm was measured using a UV–visible spectrophotometer (Hewlet Packard, model 8453, Columbia, USA). Chlorophyll content was expressed as mg Chl kg^{-1} fresh weight (fw). All measurements were made in triplicates.

2.3.6. Total phenolics content

Frozen samples of 0.5 g were homogenized with 3 mL of methanol/water (4:1 v/v) in an Ultraturrax (Janke & Kunkel, Ika-Labortechnik, Germany) at 24,000×*g* for 1 min. Next, the tubes were placed on an ice bed, in darkness and were homogenized with a vortex at time zero, after 30 min and after 1 h. Afterwards 2 mL sample was transferred in 2 × 2 mL eppendorfs and centrifuged at 15,000×*g* for 10 min at 4 °C. The supernatant was used as an extract for each sample. The amount of total phenolic compounds was determined as described previously by Singleton and Rossi (1965). A 100 μL aliquot of extract was mixed with 150 μL of Folin–Ciocalteu reagent (1:1 v/v, diluted with milli-Q-water) and incubated for 1 min before 1 mL of 75 g L^{-1} sodium carbonate (2% w/v) + NaOH

(0.4% v/v) was added. The mixture was then incubated for 1.5 h at room temperature in darkness measuring the absorbance at 765 nm (Hewlet Packard 8453, UV–Vis spectrophotometer, Columbia, USA). Total phenolics content was expressed as chlorogenic acid equivalents (ChAE) in mg kg^{-1} fw. All extracts were analyzed in triplicates.

2.3.2. Vitamin C content

Vitamin C content was determined according to the method described by Wright and Kader (1997) for the determination of ascorbic (AA) and dehydroascorbic (DHAA) acids content by HPLC. Ten grams of frozen ground leaves were added to 10 mL of extraction medium (19.2 g L^{-1} citric acid, 0.5 g L^{-1} ethylene diamine tetraacetic acid (EDTA) disodium salt, 50 mL L^{-1} methanol and 1.68 g L^{-1} NaF). The mixture was then directly homogenized for 30 s on ice using an ultraturrax (Ika-Werke, Staufen, Germany) and filtered through cheesecloth. The filtrate was collected and centrifuged at 10,500×g for 5 min at 5 °C (Heraeus Fresco 21 Thermo Electron Corporation, Germany). The filtrate was adjusted to pH 2.35–2.40, flushed through an activated Sep-Pak C18 cartridge (Waters, Milford, MA) and then filtered through a 0.45 μm filter. Afterwards, 1 mL of 1,2-phenylenediamine dihydrochloride (OPDA) solution (35 mg 100 mL^{-1}) was added to 3 mL extract for dehydroascorbic acid derivatization into the fluorophore 3-(1,2-dihydroxyethyl)furo[3,4-b]quinoxaline-1-one (DFQ). After 37 min in darkness, the samples were analyzed by HPLC (Agilent 1100 Series, Berlin, Germany). The HPLC system was equipped with a diode array working at 261 nm (L-ascorbate) and 348 nm (dehydroascorbate). Duplicates of 20 μL of each extract were injected using a stationary phase into a reverse-phase C18 μ-Bondapak ODS-5S (10 μm) stainless steel column (5 μm, 3.9 × 300 mm) (Waters μ-Bondapack, Ireland) and a guard column (Nucleosil 5 μm 100 A). The flow rate was fixed at 1 mL min^{-1} at 25 °C. Standards of L-ascorbate and dehydroascorbate (Aldrich Chemical Co., Berlin, Germany) were used. Total vitamin C content was calculated by adding AA + DHAA and results were expressed as mg vitamin C kg^{-1} fw. All extracts were analyzed in triplicates.

2.4. Statistical analysis

The experiment was a 5 × 4 bifactorial design (MAP treatment × storage time) which was analyzed using comparison among treatments carried proc mixed and Tukey's multiple comparisons of means in Statistical Analyses System 9.2. (SAS Institute, Cary, NC, USA). Statistical significance was established when $p < 0.05$. All data was previously evaluated for normality and homogeneity of variance using the proc univariate function of SAS.

3. Results and discussion

3.1. Respiration rate and gas composition within packages

The RR of Red Chard baby leaves was 30–35 mg CO_2 kg^{-1} h at 5 °C. This rate was within the range reported for mature Chard by Cantwell, Rovelo, Nie, and Rubatzky (1998), where 8.8 ± 0.7 and 40.4 ± 3.5 mg CO_2 kg^{-1} h at 0° and 10 °C respectively were observed. These differences in RR may be due to genotype, cropping systems, environmental conditions, physiological stage at harvest and storage conditions.

The initial gas partial pressures of passive MAP (Fig. 1) were 20.2 kPa O_2 and 0.03 kPa CO_2 balanced with N_2. As expected, due to the respiratory activity of leaves and the film permeability, the O_2 partial pressure decreased and the CO_2 increased. Consequently, 15.8 kPa O_2 and 4.8 kPa CO_2 within packages after 8 days at 5 °C were monitored. This equilibrium of partial pressures could be considered as recommended for fresh-cut baby leaf vegetables at 5 °C (Artés-Hernández, Escalona, Robles, Martínez-Hernández, & Artés, 2009; Nicola, Tibaldi, & Fontana, 2009). In O_2-enriched MAP treatment (Fig. 2A), gas partial pressures at 5 °C reached 6–7 kPa CO_2 and 87 kPa O_2 balanced with N_2 after 8 days. This slight difference on CO_2 partial pressure between passive and superatmospheric MAP was mainly due to the film used, which had a lower permeability and it also could be caused by the high O_2 levels which could increase levels of the RR within packages (Escalona, Verlinden, Geysen, & Nicolaï, 2005).

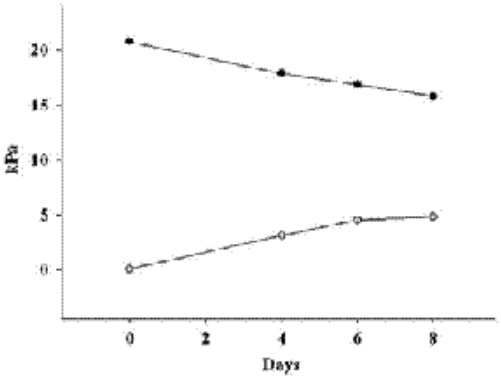

Fig. 1. Gas changes (● O_2; ○ CO_2) of fresh-cut Red Chard stored under passive MAP during storage at 5 °C. Error bars show standard deviation (SD).

N_2-enriched MAP (Fig. 2B) maintained its N_2 level throughout the shelf life over 95 kPa and the O_2 partial pressure below 1 kPa. In the He-enriched MAP treatment (Fig. 2C), He partial pressure progressively decreased within packages to 20 kPa after 8 days at 5 °C. Such loss of partial pressure was faster than that monitored for the other active MAP treatments. This can be attributed to the gas permeability of the film, the small molecular size of the atom (0.005 nm atomic radius) and the higher difference in He partial pressures inside and outside the packages. As expected, the general effect in gas changes for both gas partial pressures, was to compensate differences to achieve a gaseous equilibrium within packages. The difference of N_2O partial pressure between inside and outside the package was also high. However, N_2O-enriched MAP kept its N_2O level throughout the shelf life over 95 kPa (Fig. 2D). Although He is an inert monatomic gas, with small atomic dimension and low solubility in plant cells, N_2O has a much bigger molecular dimension and 77% solubility in vegetable cells (Couble, Fath, & Soudain, 1995) which explains this behaviour. The O_2 partial pressure was above 0.5 kPa for all treatments throughout shelf life, the theoretical limit for aerobic respiration (Saltveit, 2003).

3.2. Colour

Changes in colour parameters are shown in Table 1. Slight increases in lightness (L*) during shelf life were found as a general trend for all treatments. N_2O-enriched MAP showed the maximum increase in L* value after 8 days at 5 °C with 11% of the value monitored on the processing day (43.2 ± 2.0). In the remaining treatments, increases between 7 and 9% regarding the initial value were found. While significant differences among treatments and storage time were found, L* differences lower than 3 units are not detectable by the human eye. This is consistent with sensory results, where no differences in terms of visual appearance were detected. Rocculi, Romani, & Dalla Rosa, 2005 reported that the best colour maintenance for fresh-cut kiwifruit in terms of L* value, was an active MAP of initial 90 kPa N_2O, 5 kPa O_2 and 5 kPa CO_2. This effect could be a consequence of an indirect enzyme inhibitory

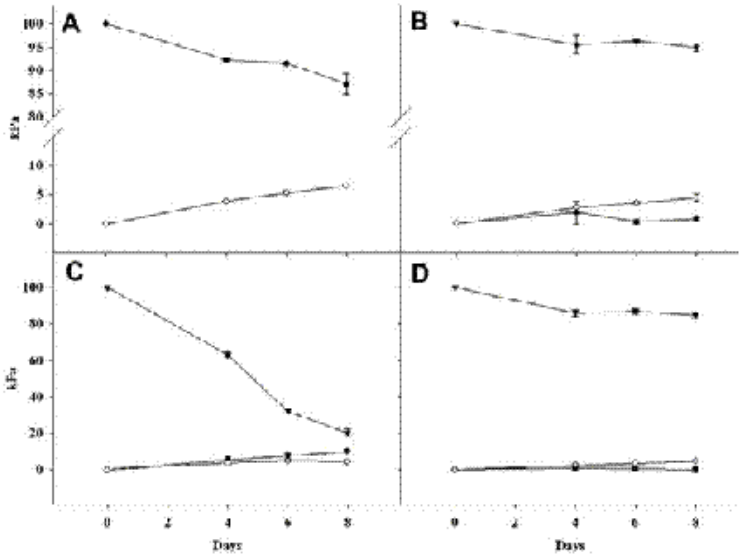

Fig. 2. Gas changes (●O_2; ○CO_2; ▼ superatmospheric gas) of fresh-cut Red Chard stored under active O_2 (A), N_2 (B), He (C) or N_2O (D) MAP during storage at 5 °C. All series are balanced with N_2 when not printed. Error bars show standard deviation (SD).

effect of N_2O. The initial Chroma value (C*) was 30.6 ± 3.1 which was kept during shelf life regardless of MAP treatment and storage time. Similar behaviour was found for Hue angle where values remained quite constant during shelf life close to 178.9 ± 0.1 for all treatments (data not shown).

3.3. Sensory evaluation

All treatments showed a moderate decrease of the overall sensory quality after 8 days at 5 °C, being scored as acceptable for fresh consumption at the limit of usability. No differences on visual appearance among treatments were observed. Red Chard baby leaves did not show browning, off-odours or off-flavours that exceeded the limit for fresh consumption. Slight dehydration symptoms were found after 8 days at 5 °C in the passive and He-enriched MAP treatments (data not shown).

Table 1
Colour parameters L*, C* changes for fresh-cut Red Chard stored under MAP during storage at 5 °C.

Colour parameters and treatments	Storage time			
	Initial	Day 4	Day 6	Day 8
L*				
Passive MAP	43.2^{a}_{1}	$46.5^{A,B}_{1,2}$	$46.3^{A,B}_{2}$	47.4^{A}_{2}
He-enriched MAP	43.2^{a}_{1}	44.2^{B}_{4}	$45.8^{A,B}_{3,2}$	46.6^{A}_{2}
O_2-enriched MAP	43.2^{a}_{1}	$45.9^{A,B}_{2}$	$46.0^{A,B}_{2}$	47.2^{A}_{2}
N_2-enriched MAP	43.2^{a}_{1}	$46.1^{A,B}_{2}$	44.5^{B}_{3}	46.7^{A}_{2}
N_2O-enriched MAP	43.2^{a}_{1}	47.2^{A}_{2}	47.3^{A}_{2}	48.6^{A}_{2}
C*				
Passive MAP	30.6^{a}_{1}	37.3^{B}_{2}	37.1^{A}_{3}	$32.8^{A,B}_{1}$
He-enriched MAP	30.6^{a}_{1}	$32.3^{A}_{1,2}$	35.2^{A}_{3}	$31.5^{A,B}_{1}$
O_2-enriched MAP	30.6^{a}_{1}	32.5^{A}_{2}	35.0^{A}_{3}	$30.7^{A,B}_{1}$
N_2-enriched MAP	30.6^{a}_{1}	$34.2^{A,B}_{2}$	34.4^{A}_{3}	30.1^{A}_{1}
N_2O-enriched MAP	30.6^{a}_{1}	37.3^{B}_{2}	37.5^{A}_{3}	33.8^{B}_{1}

Different numbers among each row denotes significant difference ($p < 0.05$). Different letter within each column denotes significant difference ($p < 0.05$). Values are mean of 30 measures.

3.4. Microbial analysis

The initial microbial load of all active MAP samples was 4.96 log CFU g^{-1} for aerobic mesophilic bacteria (Fig. 3A). This result agrees with that previously reported for fresh *Swiss* Chard (Moreira, Roura, & Del Valle, 2003). The passive MAP treatment, disinfected with 100 mg L^{-1} NaClO, showed a reduction of 0.67 log CFU g^{-1}. After 6 days at 5 °C, the microbial counts were 5.36 log CFU g^{-1}, 6.09 log CFU g^{-1} and 6.75 log CFU g^{-1} for the superatmospheric O_2, N_2-enriched and passive MAP respectively. Regarding the control samples, the O_2-enriched MAP showed a beneficial effect for inhibiting the aerobic mesophilic growth (1.38 log CFU g^{-1}). This inhibitory effect could be related with the toxicity of high O_2 concentrations to cells (Wszelaki & Mitcham, 2000), which may induce DNA and nucleoprotein damage, as well as general protein damage in microorganism (Moradas-Ferreiras, Costa, Piper, & Pagen, 1996). Compared to passive MAP no significant reductions ($p < 0.05$) for He, N_2 and N_2O-enriched MAPs in aerobic mesophilic growth were found. Our findings agree with that reported by Koseki and Itoh (2002) in which the aerobic mesophilic growth on fresh-cut lettuce and cabbage under N_2-enriched MAP for 5 days at 5 °C was not different from that under passive MAP. Regarding Enterobacteriaceae growth (Fig. 3B), the initial microbial counts were 3.01 log CFU g^{-1} and 3.79 log CFU g^{-1} for the chlorinated passive MAP and for all active MAPs respectively. A slight inhibitory effect of high O_2 levels on Enterobacteriaceae growth during shelf life was observed. Regarding control, reductions of 0.67 log CFU g^{-1} and 0.65 log CFU g^{-1} were recorded for O_2-enriched MAP on days 4 and 6. No differences were found for the remaining treatments. The disinfection with 100 mg L^{-1} NaClO of the passive MAP treatment reduced the initial load (4.64 log CFU g^{-1}) in almost 1 log CFU g^{-1} for psychrotrophic bacteria compared to the other treatments (Fig. 3C). However, slight differences between passive and He-enriched MAP throughout shelf life were found. Koseki and Itoh

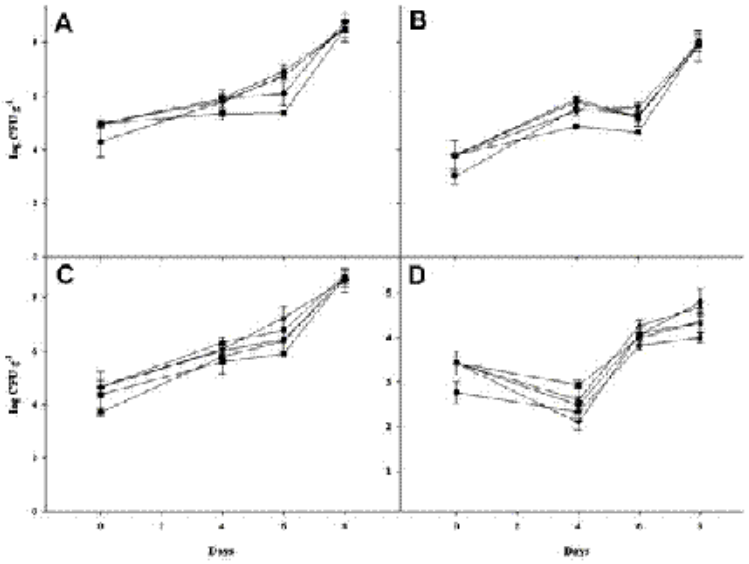

Fig. 3. Mesophilic (A), enterobacteria (B), psychrophilic (C) and yeast and moulds (D) counts (log CFU g^{-1}) of fresh-cut Red Chard stored under MAP (●Passive; ■O_2; ◆N_2; ▼He; ▲N_2O) during storage at 5 °C. Error bars show standard deviation (SD).

(2002) did not find differences on psychrotrophic growth in both passive and N_2-enriched MAP for fresh-cut lettuce after 5 days at 5 °C. High He levels favoured the psychrotrophic bacteria growth reaching 7.23 log CFU g^{-1} after 6 days at 5 °C. As in the other bacteria groups, the inhibitory effect for psychrotrophic growth of high O_2 was observed. No relevant changes in yeast and mould growth were found (Fig. 3D). Concerning *Salmonella* spp., *Listeria monocytogenes* and generic *E. coli*, no colonies were detected for any treatment on processing day according to Regulation EC 1441/2007 (2007).

3.5. Chlorophyll content

Changes of total chlorophyll (Chl *a* + Chl *b*) content in fresh-cut Red Chard baby leaves throughout 8 days at 5 °C are shown in Fig. 4. The initial total chlorophyll amount was 494 ± 17 mg Chl kg^{-1} fw. This level was similar to that reported (437 mg kg^{-1}) by Roura, Davidoch, and Del Valle (2000). The Chl *a* content represented around 80–85% of total chlorophyll content for all treatments. He-enriched MAP showed a beneficial effect for keeping the total chlorophylls content throughout shelf life when compared to N_2, N_2O, O_2-enriched MAPs and passive MAP which registered 16, 20, 26 and 21% of losses respectively. Jaime and Saltveit (2002) reported a better chlorophyll retention in broccoli florets held for 7 days at 15 °C under 2 kPa O_2 and 98 kPa He than in air since He has diffusivity characteristics which might enhance gas diffusion and reduced the concentration gradient of O_2 between the inside and outside of a commodity.

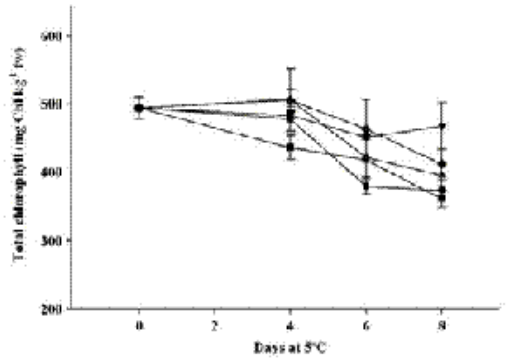

Fig. 4. Total chlorophylls content (mg Chl kg^{-1} fw) changes in fresh-cut Red Chard stored under MAP (●Passive; ■O_2; ◆N_2; ▼He; ▲N_2O) during storage at 5 °C. Error bars show standard deviation (SD).

3.6. Total phenolics content

Changes in total polyphenols content of fresh-cut Red Chard baby leaves stored up to 8 days at 5 °C under conventional and innovative active MAP are shown in Fig. 5. Total phenolics content at harvest was 613 mg ChAE kg^{-1} fw. Previous reports on extracts from mature leaves of Swiss Chard have shown a content of 8000 mg gallic acid equivalent (GAE) kg^{-1} fw (Pyo et al., 2004). The initial amount at harvest was kept between the ranges of 560–671 mg ChAE kg^{-1} fw for all treatments up to 4 days at 5 °C. The most remarkable differences were found after 6 days of shelf life under He, O_2 and N_2-enriched MAPs, where 93, 80 and 61% increases, regarding values at harvest respectively were observed. The wound-induced phenomenon in the phenolic metabolism could explain this behaviour. However, enzymatic activities such as PAL activity should be tested to corroborate the hypothesis. Thereafter, total polyphenol content gradually decreased during the remainder shelf life, registering after 8 days at 5 °C increases of 25–40% of the initial values with the exception of N_2O-enriched treatment that showed a 7% reduction. Our result about high O_2 effect for increasing total polyphenol content agrees with a recent report showing that higher than 60 kPa O_2 increased total phenolics in strawberries stored 7 days at 5 °C (Zheng, Wang, Wang, & Zheng, 2007). However, our result on He and N_2-enriched atmospheres did

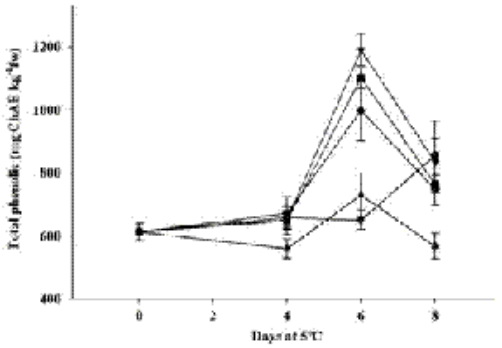

Fig. 5. Total polyphenols content (mg ChAE kg^{-1} fw) changes in fresh-cut Red Chard stored under MAP (●Passive, ■O_2, ◆N_2, ▼He, ▲N_2O) during storage at 5 °C. Error bars show standard deviation (SD).

not agree with those reporting that CA of 90 kPa He + 2 kPa O_2 and 90 kPa N_2 + 2 kPa O_2 did not delay the phenolics accumulation in fresh-cut lettuce stored at 7 °C for 4 days (Jaime & Saltveit, 2002).

3.7. Total vitamin C content

Postharvest losses of vitamin C are influenced by several factors such as temperature, humidity, atmosphere, physical damage and chilling injury (Moreira et al., 2003). Large genotypic variation, climatic conditions and cultural practices are preharvest factors responsible for the wide variation in vitamin C content of fruits and vegetables (Lee & Kader, 2000). The total vitamin C content (AA + DHAA) on processing day was 1534.1 ± 36.0 mg kg^{-1} fw (Fig. 6). This value was considerably higher than that of 450 mg kg^{-1} reported for fresh-cut Swiss Chard (Gil et al., 1998). This difference can be attributed to different plant material, maturity stage, agricultural practices, season, etc. Several authors have reported that even N_2 fertilizers, especially at high rates, seem to decrease the vitamin C concentration (Moreira et al., 2003). From the total initial content, the DHAA form represented more than 95% and the rest was the AA form. Quite similar results were found by Gil et al. (1998) for minimally processed Swiss Chard where only DHAA was detected. This could be associated with a high oxidase activity (PPO, ascorbate oxidase, cytochrome oxidase, and peroxidase) in the extracts. In all our observations we recorded a progressive decrease of vitamin C content in all treatments, although innovative active MAP treatments registered lower vitamin C losses than passive MAP. After 4 days at 5 °C, the vitamin C losses were between 8 and 15% for the active MAP treatments while passive MAP registered 50% losses. Higher reductions (50%) were observed after 8 days at 5 °C for all active MAPs and 67% for the passive MAP.

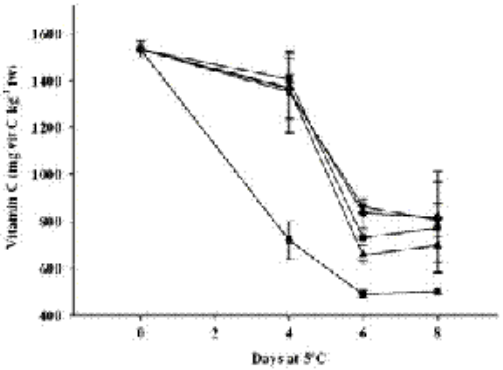

Fig. 6. Total vitamin C content (mg vit C kg^{-1} fw) changes in fresh-cut Red Chard stored under MAP (●Passive, ■O_2, ◆N_2, ▼He, ▲N_2O) during storage at 5 °C. Error bars show standard deviation (SD).

4. Conclusions

High O_2 MAP (>85 kPa O_2) showed a beneficial effect by lowering the natural microflora growth throughout 7 days at 5 °C regarding to a conventionally disinfected control stored under passive MAP. Microbial growth for He and N_2O-enriched MAPs was similar to that registered by the control. Total phenolics content at harvest increased during shelf life under innovative He, O_2 and N_2O-enriched MAPs. Such alternative treatments registered lower vitamin C losses than control. Our results suggest that He and O_2-enriched MAPs are both efficient tools for keeping overall quality of fresh-cut Red Chard baby leaves as effective as a conventionally sanitized treatment stored under passive MAP.

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CHAPTER 2

Neutral and acidic electrolyzed water as emergent sanitizers on fresh-cut Mizuna baby leaves

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Neutral and acidic electrolyzed water as emergent sanitizers for fresh-cut mizuna baby leaves

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ABSTRACT

The effects of 40, 70 or 100 mg L^{-1} free chlorine neutral and acidic electrolyzed water (NEW and AEW) during the washing and disinfection step, on quality attribute changes during shelf life of fresh-cut mizuna baby leaves, were studied. Physiological, nutritional, enzymatic, sensory, and microbial changes throughout 11 days at 5 °C were monitored. Results were compared to those reached with a conventional industrial treatment of 100 mg L^{-1} NaClO at pH 6.5 and with a control washing with deionised water. Both NEW and AEW showed an inhibitory effect on natural microflora growth and retained the main quality attributes. Total chlorophyll content was preserved after shelf life. Initial total phenolic contents ranged between 1868 and 2518 mg CAE kg^{-1} fw for AEW 40 and AEW 100 treatments respectively and slightly increased throughout shelf life. In contrast, after shelf life the total antioxidant activity recorded on the processing day decreased around 35%. Throughout shelf life EW induced an increase in catalase activity while superoxide dismutase activity decreased. Scanning electron microscopy of the leaves showed that neither NEW nor AEW affected their surface structure. To the best of our knowledge, the effects of NEW and AEW on bioactive quality parameters, as well as on antioxidant enzyme activities for fresh-cut baby leaves are first reported here. EW provides an alternative sanitising technique to NaClO for maintaining the quality of fresh-cut mizuna baby leaves up to 11 days at 5 °C.

1. Introduction

Electrolyzed water (EW), both acidic and neutral types, has been proposed as a new sanitizer for the food industry (Izumi, 1999; Liao et al., 2007; Mahmoud, 2007; Rico et al., 2008; Artés et al., 2009). The main advantage of EW is its safety. In contrast with the problems with the use of NaClO, such as skin and membrane irritation and toxicity, neutral and acidic electrolyzed water (NEW and AEW) is not corrosive to skin, mucous membranes, or organic material. In addition, when EW comes in contact with organic matter, or is diluted by tap water or reverse osmosis water, it becomes ordinary water again. Thus, it is more eco-friendly than NaClO and is not potentially harmful for human health (Huang et al., 2008). The main disadvantage is related to the rapid loss in antimicrobial activity if EW is not supplied with H^+, HOCl, and Cl_2 by electrolysis (Kiura et al., 2002). Hsu and Kao (2004) studied the effects of storage conditions on chemical and physical properties of EW and showed that pH, oxidation–reduction potential (ORP), conductivity and chloride ion concentration did not change much under the storage conditions. Total residual chlorine and dissolved O_2 decreased 24% and 21%, respectively, in 21-days closed storage and decreased 81% and 41%, respectively, in 12-days semi-open storage.

The use of AEW and NEW as disinfectants for food processing equipment has been studied (Kim et al., 2001; Park et al., 2002; Guentzel et al., 2008). Ayebah and Hung (2005) reported that AEW did not have any adverse effect on stainless steel. However, issues such as gas emission, strong acidity, metal corrosion, free chlorine content and formation of by-products need to be supported with further research.

The efficacy of NEW and AEW to reduce natural microflora as well as the main foodborne pathogens associated with a few fresh-cut plant products has been reported. The use of AEW resulted in moderate control of bacterial growth on fresh cut cilantro during storage (Wang et al., 2004). Reductions of viable aerobes by 2 log CFU g^{-1} were reached in lettuce washed with AEW (pH 2.6; ORP 1140 mV; 30 mg L^{-1} of free chlorine) for 10 min (Koseki et al., 2001). Also, AEW was effective in reducing *Escherichia coli* O157:H7 (Venkitanarayanan et al., 1999; Sharma and Demirci, 2003; Liao et al., 2007; Stopforth et al., 2008), *Salmonella* and *Listeria monocytogenes* (Venkitanarayanan et al., 1999; Fabrizio and Currier, 2003; Stopforth et al., 2008). Compared to AEW, NEW could be less aggressive in relation to the corrosion of processing equipment due to its neutral pH. NEW (pH 6.8) containing 15–50 mg L^{-1} of

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available chlorine was effective as a disinfectant for fresh-cut vegetables without causing discolouration: NEW did not affect tissue pH, surface colour or general appearance (Izumi, 1999). Abadias et al. (2008) demonstrated that the bactericidal activity of diluted NEW (50 mg L^{-1} free chlorine, pH 8.6) against *E. coli* O157:H7, *Salmonella*, *Listeria innocua* and *Erwinia carotovora* on fresh-cut lettuce was similar to that with NaClO (120 mg L^{-1} free chlorine).

A theory for inactivation of bacteria by EW was reported by Liao et al. (2007) on *E. coli* O157:H7. The inactivation mechanism proposed that ORP could first affect and damage the redox state of glutathione disulfide–glutathione couple (GSSG/2GSH), and then penetrate the outer and inner membranes of *E. coli* O157:H7 resulting in the bacteria necrosis.

The application of EW in the food industry has been recently reviewed as a disinfectant for food processing equipment, vegetables, fruit, poultry, meat and seafood (Huang et al., 2008). However, there is little information available on the effects of NEW and AEW on the main quality attributes in fresh-cut vegetables.

Yellowing or green colour loss is a natural phenomenon during plant senescence as a consequence of chlorophyll degradation. Several studies have reported the activity of this pigment with the prevention of cancer (Egner et al., 2001, 2003; De Vogel et al., 2005) as well as their absorption by humans (Ferruzzi and Blakeslee, 2007). Polyphenols contribute to flavour, natural food pigments and stress resistance of vegetables (Lule and Xia, 2005). Phenolic compounds can act as antioxidants (Shahidi and Naczk, 1995) by interfering with oxidation processes through chain-breaking reaction activities (primary oxidation) or through scavenging of free radicals (secondary oxidation) (Gordon, 1990). The antioxidant properties of phenolic compounds stimulate the need to design strategies to enhance its content in plant tissues (Cisneros-Zevallos, 2003). Antioxidants scavenge reactive oxygen species (ROS) which can cause cell damage in plant tissues. ROS are generated in normal metabolic processes as by-products of cell metabolism (Reyes et al., 2007). The antioxidant activity could be attributed mainly to vitamin C. Phenolic compounds and vitamin C are the major antioxidant sources of *Brassica* vegetables, due to their high content and high antioxidant activity. On the contrary, lipid-soluble antioxidants (carotenoids and vitamin E) are responsible for up to 20% of the total antioxidant activity (Podsedek, 2007). Antioxidant systems in plants prevent or mitigate the membrane peroxidation resulting from ROS under abiotic stress conditions (Korkmaz et al., 2010). SOD is a group of metalloenzymes that protect cells from oxidative damage by dismutating superoxide radicals to molecular O_2 and H_2O_2. Then, the latter product, a potentially toxic compound, is converted to water by a number of enzymes such as catalase (CAT), ascorbate peroxidase (APX) and peroxidase (POD) (Kochhar et al., 2003). SOD enzymes are located in the chloroplast, mitochondria, cytoplasm and peroxisomes and operate as the first line of defence against ROS (Liao et al., 2007). The general deactivation found for SOD activity during storage could be due to the oxidation of the amino acids and/or disulfite bonds, which are involved in the active site of SOD.

The aim of this study was to investigate the effects of NEW and AEW as emergent sanitizers on the overall quality of fresh-cut mizuna baby leaves including physiological, microbial, nutritional and sensory changes, compared to a conventional disinfection used in the industry with sodium hypochlorite (100 mg L^{-1} NaClO at pH 6.5) and versus a control washing with deionised water. As far as we know, the effects on bioactive quality parameters, as well as on antioxidant enzyme activities, of NEW and AEW during shelf-life of fresh-cut baby leaves have not been earlier reported.

2. Materials and methods

2.1. Plant material

Mizuna (*Brassica rapa* cv. Japonica) baby leaves were cultivated in the open air under the Mediterranean climate of the 'Campo de Cartagena' (Murcia, Spain) and mechanically harvested at the commercial development stage. Immediately after harvesting, the leaves were transported in about 30 min to the Pilot Plant of the research group in the Technical University of Cartagena (UPCT) where they were pre-cooled by air at 5 °C.

2.2. Production of electrolyzed water

For EW production, an Envirolyte EL 400 device (Aquaticja, Madrid, Spain) was used. Its design of the diaphragmatic cell ensures that the electrolyte used in the production of activated solutions has a maximum contact area with the electrodes. The EW is generated using a continuous supply of dilute salt water (10% NaCl in tap water). Two types of processing water with different characteristics are generated. An electrolyzed basic solution (pH 11 and ORP < −800 mV) is produced at the cathode side, and an electrolyzed acid solution (pH 3 and ORP > 1100 mV) is produced at the anode side.

To obtain NEW, it was drained from the anode and from the cathode and mixed in correct proportions. NEW had a pH value 7.0 ± 0.1 containing 410 mg L^{-1} of free chlorine. AEW had a pH value 3.1 ± 0.1 (drained from the anode) containing 260 mg L^{-1} of free chlorine. To obtain the desired concentrations of 40, 70 and 100 mg L^{-1} of free chlorine for both NEW and AEW, an appropriate proportion of distilled water was mixed with the original solutions. Free chlorine and pH were determined using a photometer (Hi 96771, Hanna Instruments, Eibar, Spain) and pH-meter (Basic 20, Crison, Barcelona, Spain) respectively, for all solutions.

2.3. Sample preparation, treatments and storage conditions

Mizuna baby leaves (*B. rapa* var. Japonica) were processed in a disinfected room at 8 °C. Leaves with defects such as yellowing, decay, cuts and bruising were discarded. The raw material was pre-washed for 1 min with tap water at 5 °C to remove traces of soil and organic matter.

NEW and AEW each in concentrations of 40, 70 and 100 mg L^{-1} of free chlorine, were prepared. A standard industrial disinfection treatment with NaClO (100 mg L^{-1}, pH 6.5) was also prepared. As control, deionised water was used. All washing treatments lasted 2 min at 5 °C and were followed by a 1 min rinsing step with tap water. Then, leaves were manually spin-dried to eliminate excess water. An amount of 40 g of leaves was placed in polypropylene (PP) trays of 1500 mL thermally sealed at the top with a bi-oriented polypropylene (BOPP) film of 50 μm thickness (Plásticos del Segura S.L., Murcia, Spain) to generate a passive modified atmosphere package (MAP). The permeability of BOPP was 2004 mL O_2 m^{-2} d^{-1} atm^{-1} and 3624 mL CO_2 m^{-2} d^{-1} atm^{-1} at 5 °C and 60% RH. Three replicates of one basket per treatment and storage duration (processing day and after 4, 7 and 11 days of shelf life) were prepared and stored in a dark cold room at 5 °C. This temperature was selected as the maximum limit recommended and it is most commonly used for fresh-cut plant commodities throughout its commercial distribution and retail sale.

2.4. Analysis and determinations

2.4.1. Respiration rate and gas composition within packages

Gas analyses were determined with a gas chromatograph (GC) (Thermo Finnigan Trace GC, Milan, Italy), equipped with a ther-

mal conductivity detector (150 °C), oven (ramp from 40 °C to 90 °C), injector (150 °C) and with a Porapack-N 80/100 column. He (20 mL min^{-1}) was the carrier gas. Calibration of CO_2 and O_2 was done with known standards from gas cylinders (Air liquid S.L., Murcia, Spain).

2.4.1.1. Respiration rate. The respiration rate (RR) of the leaves was determined at 5 °C by using a closed system. Three replicates of 20 g leaves were placed within 750 mL glass jars at 5 °C up to 7 days. In order to avoid CO_2 accumulation higher than 0.03 kPa (Watada et al., 1996) and to maintain a high RH within jars, a continuous flow of 20 mL min^{-1} humidified air was applied through the glass jars. The increases in CO_2 were daily monitored after closing the jars for 3 h. After taking the samples, the jars were continuously flushed again with the humidified air until the next sampling time. Headspace gas samples (1 mL) were withdrawn from the jars with a gas-tight syringe and analyzed in the GC. Three replicates were made for each treatment and evaluation period.

2.4.1.2. Gas composition within packages. Gas composition (O_2 and CO_2) within the packages was monitored on the processing day and after 1, 2, 4, 7 and 11 days of storage. Headspace gas samples (1 mL) were withdrawn from the packages using a gas-tight syringe and analyzed in the GC. Three replicates were made for each treatment and evaluation period.

2.4.2. Microbial analysis

Standard enumeration methods were used to determine the microbial growth. Three random samples were taken on each evaluation time. A 10 g sample of leaves was homogenized in 90 mL of sterile peptone saline solution (pH 7) (Scharlau Chemie SA, Barcelona, Spain) for 1 min in a sterile stomacher bag (Model 400 Bags 6141, London, UK) using a Masticator (Colwort Stomacher 400 Lab, Seward Medical, London, UK). In order to determine the enumeration of each microbial group (mesophilic, enterobacteria, psychrotrophic, lactic acid bacteria, yeasts and moulds), ten-fold dilution series were prepared in 9 mL of sterile peptone saline solution. Mesophilic, enterobacteria, lactic acid bacteria and psychrotrophic were pour-plated. Yeast and mould were spread-plated. The following media and incubation conditions were used: plate count modified agar (Scharlau Chemie, Barcelona, Spain) for mesophilic and psychrotrophic aerobic bacteria, incubated at 30 °C for 48 h and at 5 °C for 7 days respectively; violet red bile dextrose agar (Scharlau Chemie, Barcelona, Spain) for enterobacteria, incubated at 37 °C for 48 h; de man-rogosa-sharpe agar (Scharlau Chemie, Barcelona, Spain) for acid lactic bacteria, incubated at 30 °C for 48 h and potato dextrose agar base (Scharlau Chemie, Barcelona, Spain) with 100 mg L^{-1} oxytetracycline (Sigma Chemical Co., St. Louis, MO, USA) for yeasts and moulds, incubated for 3–5 days at 22 °C. All microbial counts were reported as log colony forming units per gram (log CFU g^{-1}). The presence of *Salmonella* spp., *L. monocytogenes* and generic *E. coli* was evaluated according to the European legislation for fresh-cut vegetables (Regulation EC 1441/2007, 2007). All analyses were made in triplicates.

2.4.3. Sensory evaluation

A seven-person panel (aged 24–62) trained in sensory quality analyses performed the evaluation. Before running the experiments a consensus was reached between the panellists in order to select those attributes that better describe sensory changes. Sensory quality was evaluated on the processing day and after 4, 7 and 11 days of shelf life at 5 °C. Dehydration, browning, off-odours and off-flavours were scored on a five-point scale of damage incidence and severity (1 = none, 2 = slight, 3 = moderate, limit of usability, 4 = severe and 5 = extreme). Visual appearance and overall quality was evaluated by using a nine-point scale (1 = extremely poor, 5 = fair, limit of usability, 9 = excellent).

2.4.4. Chlorophyll content

For tissue preparation, about 25 g of leaves from each treatment were frozen in liquid N_2 and ground using a mincer (IKA, A 11 basic, Berlin, Germany) at 28,000 × *g* for 10 s, and stored at −80 °C. The sample preparation for chlorophyll determination was according to Smith and Benitez (1955). The total chlorophyll pigments were extracted with hexane. A 0.5 g sample of frozen shredded leaves was mixed with 9 mL of hexane and 15 mL of a mixture of methanol:acetone (1:2). The extraction was carried out for 5 h at 5 °C in darkness. Then, samples were shaken at 200 × *g* every 15 min by using a vortex (Velp Scientifica, Zx^3, Milano, Italy). After the incubation time, 25 mL of a solution of 1 M NaCl was added. Subsequently the samples were shaken again and then centrifuged for 30 min at 2800 × *g* at 4 °C. After centrifugation, 1 mL of the supernatant was removed and introduced into a quartz cuvette (Hellma GmbH & Co. Müllheim, Germany). The equations developed by Wellburn (1994) were used to determine the individual levels of both chlorophyll *a* (Chl_a) = $10.05A_{662} - 0.76CA_{644}$ and chlorophyll *b* (Chl_b) = $16.37A_{644} - 3.14A_{662}$ where total chlorophylls was calculated as $Chl_a + Chl_b$. The absorbance (*A*) at 662 and 644 nm was measured using a UV-visible spectrophotometer (Hewlett Packard, model 8453, Columbia, USA). Chlorophyll content was expressed as mg kg^{-1} fresh weight (fw). All measurements were made in triplicates.

2.4.5. Total phenolic content

Frozen samples of 0.5 g were homogenized with 3 mL of methanol/water (4:1 v/v) in an Ultraturrax (Janke & Kunkel, Ika Labortechnik, Stauten, Germany) at 24,000 × *g* for 1 min. Then, the tubes were placed on an ice bed, in darkness and were homogenized with a vortex at time zero, after 30 min and after 1 h. Afterwards 2 mL of sample was transferred into two 2 mL eppendorf tubes and centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant was used as the extract for each sample. The amount of total phenolic compounds was determined as described by Singleton and Rossi (1965). A 100 μL aliquot of extract was mixed with 150 μL of Folin–Ciocalteu reagent (1:1 v/v, diluted with Milli-Q-water) and incubated for 1 min before 1 mL of 75 g L^{-1} sodium carbonate (2% w/v) + NaOH (0.4% w/v) was added. The mixture was incubated for 1.5 h at room temperature in darkness, measuring the absorbance at 765 nm (Hewlett Packard 8453, UV-vis spectrophotometer, Columbia, USA). Total phenolic content was expressed as chlorogenic acid equivalents (CAE) in mg kg^{-1} fw. All extracts were analyzed in triplicates.

2.4.6. Total antioxidant activity

The antioxidant activity of the fresh-cut mizuna leaves was based on the evaluation of the free radical scavenging capacity according to Brand-Williams et al. (1995). A solution of 0.7 mM 2,2-diphenyl-1-picrylhydrazil radical (DPPH) in methanol was prepared. An aliquot of 25 μL of the extract obtained from the preparation of phenolic compounds was added to 975 μL of this solution. The mixture was incubated in darkness for 1 h at room temperature. The antioxidant activity was measured by decreasing the absorbance at 517 nm (Hewlett Packard 8453, UV-vis spectrophotometer, Columbia, USA). Results were expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) kg^{-1} fw. All measurements were made in triplicates.

2.4.7. Enzymatic activity

2.4.7.1. Enzyme extract preparation. Enzymes were extracted according to Kang and Saltveit (2001) method with slight modi-

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2.4.7. Enzymatic activity

2.4.7.1. Enzyme extract preparation. Enzymes were extracted according to Kang and Saltveit (2001) method with slight modi-

fications. An amount of 4 g of frozen (−80 °C) ground mizuna baby leaves, were homogenized in 4 mL of a chilled solution (4 °C) containing 0.05 M Tris–HCl Buffer (pH 7.5), 3 mM $MgCl_2$ and 1 mM ethylene diamine tetraacetic acid (EDTA), for 1 h with an orbital shaker at 250 × g. The homogenate was centrifuged at 20.000 × g for 20 min at 4 °C. The supernatant was used as enzyme extract.

2.4.7.2. Superoxide dismutase (SOD) activity. SOD activity was assayed by its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Dhindsa et al. (1981) with slight modifications. A 96 polystyrene flat-bottom well plate (Greiner Bio-one, Frickenhausen, Germany) was loaded with 351 μL reaction contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 6 μL of enzyme extract. Riboflavin was added last and the plate was placed 30 cm below a light bank consisting of two 7.5 W fluorescent lamps for 20 min. A non-irradiated reaction mixture did not develop colour and served as the control. The absorbance by the reaction mixture was read at 560 nm using a multiscan plate reader (Tecan Infinite M200, Männedorf, Switzerland) at time 0 and 1 h. SOD activity was calculated per g of fresh sample and expressed as a percentage of the control. All measurements were made in triplicate.

2.4.7.3. Catalase (CAT) activity. CAT activity was assayed by measuring the rate of disappearance of H_2O_2 using the method of Maehly and Chance (1959) with slight modifications. The reaction mixture was properly loaded on a 96 UV star flat-bottom well plate (Greiner Bio-one, Frickenhausen, Germany) containing 349 μL of 50 mM phosphate buffer (pH 7.4), 13 μL of 1% H_2O_2 and 6 μL enzyme extract, diluted to 60% to keep measurements within the linear range of the analysis. The H_2O_2 decrease was monitored as a decline in absorbance at 240 nm using a multiscan plate reader (Tecan Infinite M200, Männedorf, Switzerland) every 10 min for 1 h. CAT activity (ΔA/min) was calculated by the initial velocities method from the linear portion of the curves. One unit of activity (*u*) was defined as a decrease in absorbance of 0.001 min^{-1} g^{-1} fw. All measurements were made in triplicates.

2.4.8. Scanning electron microscopy (SEM)

The leaf tissue was dehydrated in a graded acetone series (from 15 to 100%, v/v) according to Escalona et al. (2010). 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 aluminum discs and coated with gold in a Sputter Coater (SC7640, Quorum Technologies, East Sussex, England) to make their surfaces electrically conductive, and were finally observed with a scanning electron microscope (S-3500N, Hitachi, Ratingen, Germany). Control, NaClO, NEW 100, AEW 100 and two EW extreme treatments (NEW 420 and AEW 240), prepared exclusively for SEM analyses, were inspected at 300× and 2000× zoom.

2.4.9. Statistical analysis

The experiment was an 8 × 4 bifactorial design (washing treatment × storage time) which was subjected to analysis of variance (ANOVA) using Statgraphics Plus (version 5.1) software. Mean values were subjected to the least significant difference test (LSD) at $P < 0.05$. Figures represent mean values ($n = 3$) ± SD.

3. Results and discussion

3.1. Respiration rate and gas composition within packages

The RR of untreated and treated mizuna leaves was monitored for up to 7 days at 5 °C. After 5 days the RR was quite stable for all

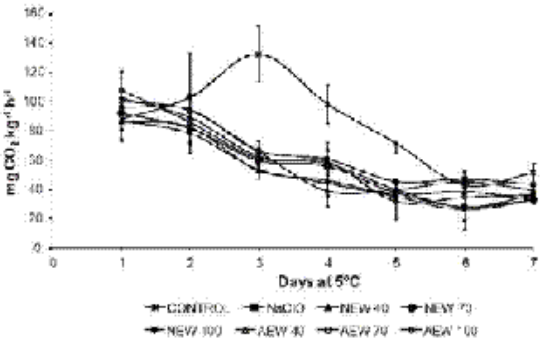

Fig. 1. Respiration rate of fresh-cut mizuna baby leaves washed under several EW treatments and stored up to 7 days at 5 °C. Data represent means of three replicates ± standard deviation (SD).

treatments at about 40 mg CO_2 kg^{-1} h^{-1} (Fig. 1). After 7 days minor differences among treatments were found. The RR of control leaves was slightly higher than that reached for the remaining treatments. Our results agree with those from Rico et al. (2008) showing that NEW decreased the RR of minimally processed lettuce which might be related with the reduction in microbial spoilage. A lower RR generally implies lower microbial spoilage, slower rate of deterioration and less loss of quality (Cliffe-Byrnes and Beirne, 2005).

The initial gas partial pressure within packages was air (21.9 kPa O_2 and 0.03 kPa CO_2). As expected, due to the respiratory activity of leaves and the film permeability, the O_2 partial pressure decreased and the CO_2 increased (Fig. 2). Gas partial pressure within packages after 11 days at 5 °C was 16.1 kPa CO_2 and 3.5 kPa O_2, which can be considered as recommended for fresh-cut baby leaf vegetables during shelf life (Allende et al., 2006; Conte et al., 2008; Artés-Hernández et al., 2009; Nicola et al., 2009).

3.2. Microbial analysis

The initial microbial load was 3.92 log CFU g^{-1} for aerobic mesophilic bacteria (Fig. 3A). This result agrees with that recently reported for fresh-cut mizuna baby leaves (Martínez-Sánchez et al., 2008a). Regarding the control treatment, after washing with chlorinated water, as well as with NEW and AEW, total mesophilic counts were slightly reduced (<1 CFU log unit). These results agree with those reported by Abadias et al. (2008) where reductions of

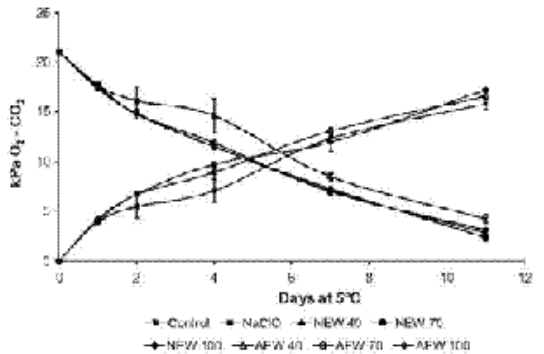

Fig. 2. Gas changes within packages of fresh-cut mizuna baby leaves washed under several EW treatments and stored up to 11 days at 5 °C. Data represent means of three replicates ± standard deviation (SD).

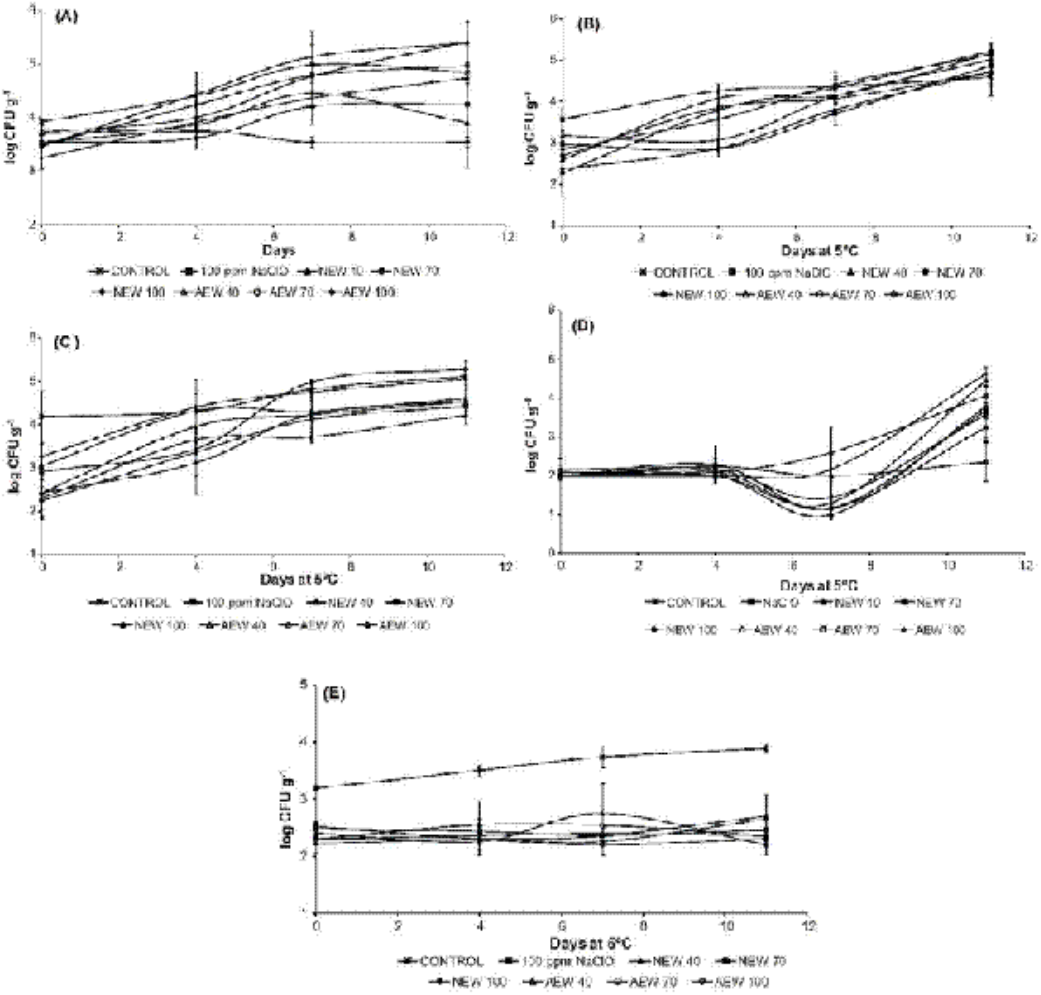

Fig. 3. Mesophilic (A), enterobacteria (B), psychrophilic (C), LAB (D) and yeast and moulds (E) counts (log CFU g^{-1}) of fresh-cut mizuna baby leaves washed under several EW treatments and stored up to 11 days at 5 °C. Data represent means of three replicates ± standard deviation (SD).

1 CFU log unit were found for fresh cut iceberg lettuce and four season salad (mixed of iceberg lettuce, carrot and cabbage) treated with NEW (pH 8.6, ORP 722 mV, 52 mg L^{-1} of free Cl) during 3 min. In the same way our results agree with those found in fresh-cut cilantro treated with AEW (pH 2.4, ORP 1130 mV, 16.8 mg L^{-1} free Cl) for 5 min (Wang et al., 2004). Both NEW 40 and NEW 70 treatments inhibited the microbial growth throughout the shelf-life at 5 °C reaching a final load of 3.90 and 3.54 log CFU g^{-1} respectively. NaClO treatment showed a good sanitizing effect on mesophilics, but lower than that of NEW 40 and 70. Izumi (1999) reported reductions on total microbial counts ranging from 0.6 to 2.6 log CFU g^{-1} on carrots, bell peppers, spinach, Japanese radish and potatoes under the effect of EW (pH 8.8, 15–50 mg L^{-1} available Cl). During shelf life, AEW treatments had a lower microbicidal effect on fresh-cut mizuna leaves compared to the NEW and NaClO treatments. However, Koseki et al. (2001) found reductions on total viable aerobes by 2 log CFU g^{-1} for lettuce treated with AEW (pH 2.6, ORP 1140 mV, 30 mg L^{-1} available Cl).

Regarding *Enterobacteriaceae* (Fig. 3B), NaClO treatment showed on the processing day a reduction of 0.6 log CFU g^{-1} compared to the control treatment. Both AEW and NEW treatments (except NEW 40) showed a similar microbiocidal effect than NaClO. Reductions of the initial enterobacteria load of 1.3, 1.2 and 1 log CFU g^{-1} for AEW 70, NEW 100 and AEW 40 respectively were found. These reduction levels are of a similar range to those reached by washing fresh-cut lettuce for 1 min in electrolyzed oxidizing water (EOW) (3.62 mg free oxidants L^{-1}) resulting in 1.3 log CFU g^{-1} of *Enterobacteriaceae* (Ongeng et al., 2006). Although the initial microbicide effect of EW treatments on mizuna leaves was higher than that with NaClO, after 4 and 7 days at 5 °C the enterobacteria load for all EW types was above the level monitored in NaClO treatment. A hypothesis for that behaviour is that AEW and NEW might cause superficial damage on the leaves, promoting the microbial growth rate. Washing for 5 min with AEW (pH 2.4, ORP 1130 mV, 16.8 mg L^{-1} free Cl) fresh-cut cilantro increased the electrolyte leakage compared to chlorinated water (50 mg L^{-1} free chlorine, pH 6.5) and tap water.

which could indicate a cilantro tissue damage (Wang et al., 2004; Gómez-López et al., 2007).

Differences on the psychrophilic group on the processing day were found (Fig. 3C). Regarding the control NaClO, AEW 40, and NEW 40 treatments showed reductions of 1.95, 1.89 and 1.77 log CFU g^{-1} respectively. The increase of microbial growth throughout shelf life was faster for the control and NEW than for AEW and NaClO treatments. After 7 days at 5 °C, total aerobic mesophilic and psychrophilic bacteria reached a stationary phase which was not increased throughout the shelf life. Similar results were found by Gómez-López et al. (2007) for fresh-cut cabbage treated with NEW. After shelf life, no differences between NaClO and AEW were found. Therefore, they were the best treatments for controlling psychrophilic bacteria growth.

The lactic acid bacteria (LAB) growth is related to the CO_2 and O_2 partial pressure. No significant differences on LAB counts at day 0 for all treatments were found (Fig. 3D) where around 2 CFU g^{-1} was found for all treatments. However, Ongeng et al. (2006) reported a reduction of 1.2 log CFU g^{-1} washing for 1 min with EOW (2.8 mg free oxidants L^{-1}) compared to a control washing treatment with tap water. During shelf life, the LAB load for AEW 70 was quite constant and NEW and NaClO had similar effects. AEW 70 showed the best disinfectant effect against LAB.

Regarding yeast and moulds growth (Fig. 3E), all disinfectant washing solutions reduced the initial load (3.19 log CFU g^{-1}) in 1 log CFU g^{-1} compared to the control and these counts were maintained constant during shelf life.

The presence of *Salmonella* spp., *L. monocytogenes* and generic *E. coli* was also studied. No colonies were detected for any treatment on the processing day on the raw material after disinfection treatments, this being safe according the EC legislation (Regulation EC 1441/2007, 2007).

Small differences among NaClO, NEW and AEW treatments in reduction of microorganisms were found. NEW had a higher effect against enterobacteria and mesophilic bacteria, and AEW for psychrotrophic and LAB. The only two factors that could explain this difference would be the ORP and the pH. Further research is needed to understand which factor has the main influence on each microorganism group.

3.3. Sensory evaluation

All treatments showed a slight decrease of their overall sensory quality after 7 days at 5 °C while still being acceptable for consumption. No noticeable differences in visual appearance, browning, dehydration, off-odours and off-flavours among treatments were found. The overall sensory quality reached the limit of usability for all treatments after 11 days at 5 °C (data not shown); this then is the shelf life established under our experimental conditions.

3.4. Chlorophyll content

The initial chlorophyll amount was around 95 mg kg^{-1} fw. The Chl *a* and Chl *b* represented around 50% each one from the total content (Fig. 4). No significant differences were found among treatments on the processing day neither after 11 days at 5 °C. In general, the initial amount was maintained throughout shelf life in a range between 95 and 110 mg Chl kg^{-1} fw. This indicates that the total chlorophyll content was not affected by the oxidizing sanitizers used in this study. MAP storage at 5 °C inhibited the natural degradation of chlorophyll. Changes monitored in the a^* parameter can be associated with degradation of chlorophyll pigments. Wang et al. (2004) did not find a difference in a^* colorimetric parameter during shelf life of fresh-cut cilantro treated with AEW. However, a^* values increased during storage at 4 °C in fresh-cut lettuce, with no significant differences between chlorine and NEW treatments (Rico et al., 2008).

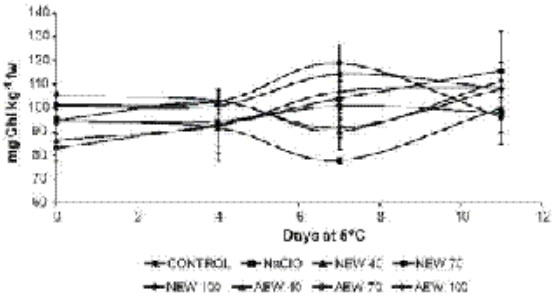

Fig. 4. Total chlorophyll content changes in fresh-cut mizuna baby leaves washed under several EW treatments and stored up to 11 days at 5 °C. Data represent means of three replicates ± standard deviation (SE).

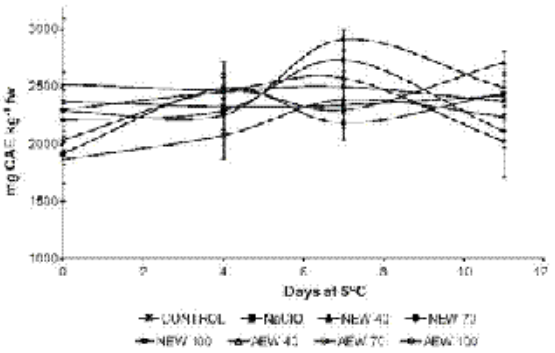

Fig. 5. Total polyphenols content changes in fresh-cut mizuna baby leaves washed under several EW treatments and stored up to 11 days at 5 °C. Data represent means of three replicates ± standard deviation (SE).

3.5. Total phenolic content

Total phenolic content of mizuna baby leaves at harvest was 2371 mg GAE kg^{-1} fw for control treatment (Fig. 5), which was higher than that of 990 mg kg^{-1} fw found by Martínez Sánchez et al. (2008b). The different polyphenols contents in *Brassica* veg-

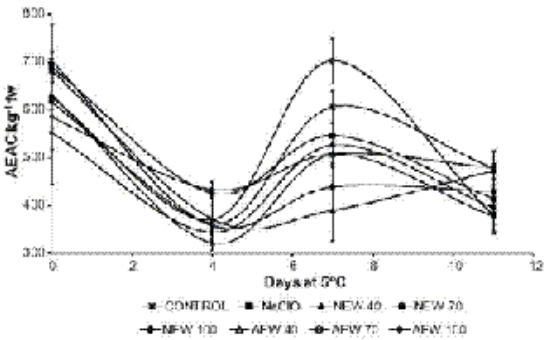

Fig. 6. Total antioxidant activity changes in fresh-cut mizuna baby leaves washed under several EW treatments and stored up to 11 days at 5 °C. Data represent means of three replicates ± standard deviation (SE).

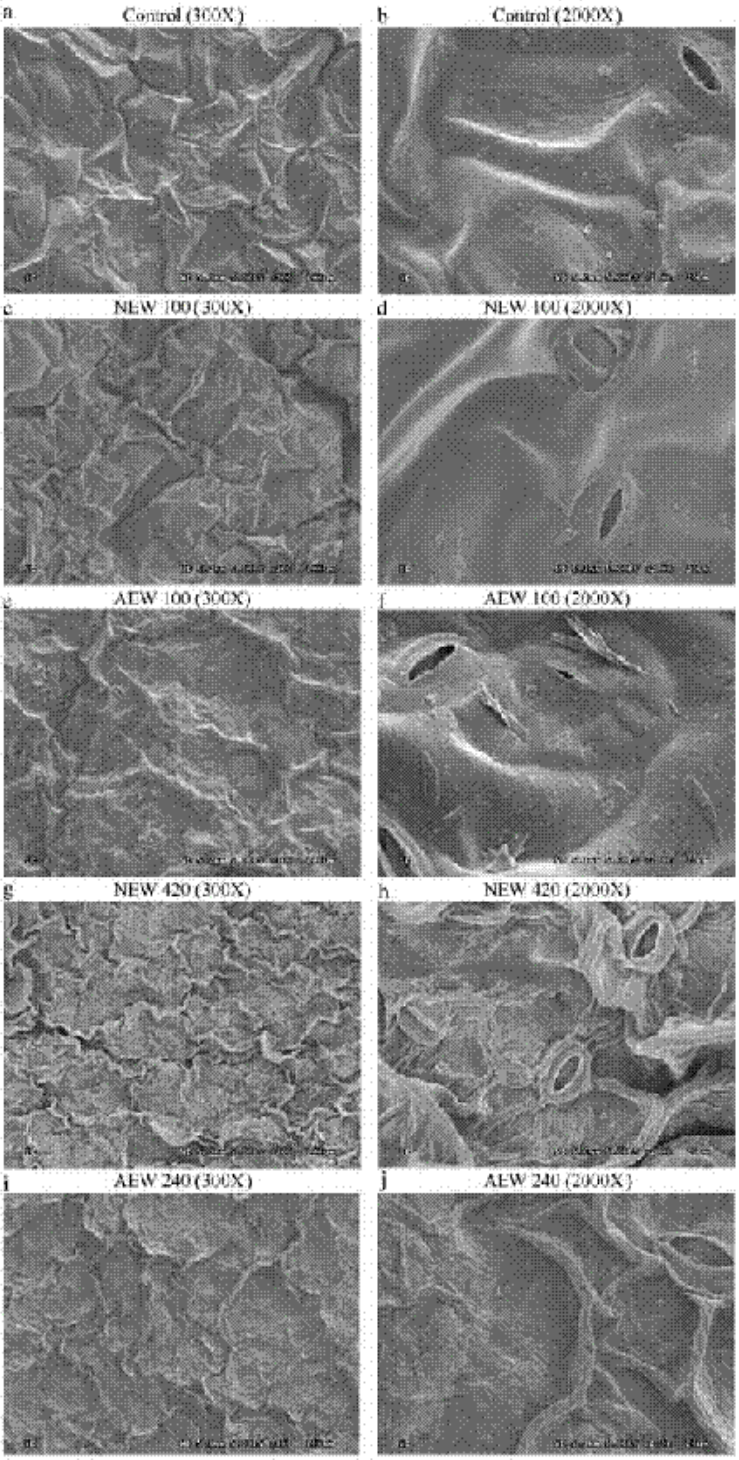

Fig. 7. Scanning electron microscope photographs (300× and 2000×) of fresh-cut mizuna baby leaves just after washing with 100 mg L^{-1} NaClO, NEW 100, AEW 100 and NEW 420 and AEW 240 as extreme EW treatments.

Table 1
Superoxide dismutase and catalase activity changes in fresh-cut mizuna baby leaves washed under several EW treatments and stored up to 11 days at 5 °C.

	Processing day	After 4 days at 5 °C	After 7 days at 5 °C	After 11 days at 5 °C
SOD activity[a] (%control g^{-1} fw)				
Control	140	116	97	94
NaClO	136	110	104	103
NEW 40	129	107	104	96
NEW 100	146	117	121	108
AEW 40	104	103	114	104
AEW 100	135	114	112	119
CAT activity[b] ($u g^{-1}$ fw)				
Control	787	2230	1367	3856
NaClO	786	2006	1404	3086
NEW 40	1022	2535	1840	3954
NEW 100	477	2337	1717	3023
AEW 40	711	2125	1764	3427
AEW 100	919	2749	2549	3560

[a] SE = 41 SD ($P \leq 0.05$) = 12
[b] SE = 110 LSD ($P \leq 0.05$) = 312

etables may be influenced by several factors, including genetic and environmental influences, growing period, and maturity stage at harvest (Naczk and Shahidi, 2006; Singh et al., 2007). Martínez-Sánchez et al. (2008b) found that the main phenolic compounds in mizuna baby leaves are isorhamnetin 3-O-glucoside and sinapic acid, each one being 23% of the total polyphenols content. On the processing day no important differences among treatments were found. The initial total phenolic amount ranged between 1858 and 2518 mg CAE kg^{-1} fw for AEW 40 and 100 treatments respectively. The general trend throughout 11 days at 5 °C was to slightly increase the initial content with no remarkable differences among treatments, except for NEW 40 which reached the highest amount with an increase of 33% after shelf life.

3.6. Total antioxidant activity

The total antioxidant activity on the processing day was 703 mg AEAC kg^{-1} fw for control leaves (Fig. 6), which was lower than 1400 mg TEAC (total equivalents of antioxidant capacity) kg^{-1} fw reported for mizuna leaves (Martínez-Sánchez et al., 2008b). This amount indicates that mizuna is a good natural source of antioxidants (Naczk and Shahidi, 2006; Singh et al., 2007). No difference in initial total antioxidant activity among treatments was found, except that for NEW 100 and AEW 100 which showed the lower levels with 585 and 553 mg AEAC kg^{-1} fw respectively.

The general trend in the total antioxidant activity up to the 4th day of shelf life was to gradually decrease the initial values (40–45%) but after 7 days a slight increase was generally found. This fact may be due to the combined effect of processing steps and chilling storage under MAP.

After 11 days at 5 °C, a general reduction of 30–40% of the initial total antioxidant activity was found. This fact might be due to vitamin C and carotenoid losses during shelf life but further studies down this path should be conducted. After shelf life no remarkable differences were found. It is noticeable that NEW treatments were generally less aggressive than AEW for maintaining initial total antioxidant activity after shelf life, and this might be due to its strong oxidizing activity.

3.7. Antioxidant enzyme activities

Mizuna baby leaves washed with both EWs generally showed a higher SOD activity than that registered for control leaves after shelf life (Table 1). SOD activity on the processing day ranged between 129 and 146 (%control g^{-1} fw) just after washing with NEW 40 and NEW 100 respectively. The general trend was to decrease its value for all treatments during shelf life, reaching reductions from 17 to 30% (after 7 days at 5 °C) and from 12 to 36% (after 11 days at 5 °C) for AEW 100 and AEW 40 respectively. Except for AEW 100, all the remaining washing treatments showed from 25 to 35% reductions of the initial SOD activity. Rico et al. (2008) reported a similar decrease in polyphenol oxidase activity in fresh-cut lettuce treated with NEW (120 mg L^{-1} free Cl).

CAT activity on the processing day ranged between 477 and 1022 ($u g^{-1}$ fw) for NEW 100 and NEW 40 respectively (Table 1). In contrast with this enzyme activity, the trend was to increase its value throughout shelf life for all treatments. Such increases ranged after 4 days at 5 °C from 2 to 5-fold the initial values for NEW 40 and NEW 100 respectively. However, after 11 days, 4–6 fold increase respectively were recorded. Many abiotic stresses have been reported to increase CAT activity, as Kang and Saltveit (2001) demonstrated for cucumber seedling radicles.

3.8. Scanning electron microscopy (SEM)

After studying the SEM pictures, no substantial differences at 300× or 2000×, on the surface structure of leaves just after washing, among control (Fig. 7a and b), NEW 100 (Fig. 7c and d) and AEW 100 (Fig. 7e and f) treatments were observed. Similar observations were reported by Koseki et al. (2001) on lettuce leaves treated with AEW (pH 2.6; ORP 1140 mV; 30 mg L^{-1} of available chlorine). In the same way, Rico et al. (2008) did not find structural differences by cryo-scanning electron microscopy on fresh-cut lettuce treated with 120 mg L^{-1} chlorine, NEW 120, NEW 60 and NEW 12 after 1 day at 4 °C.

Extreme EW treatments in our study caused severe structure surface damages such as cracks and dehydration with consequent turgor loss, as was found for NEW 420 and AEW 240 (Fig. 7g and h, and Fig. 7i and j respectively).

4. Conclusions

As a main conclusion, washing with EW seems to be a promising disinfection technique, as effective as the conventional disinfection with 100 mg L^{-1} NaClO. Both AEW and NEW (40 or 100) showed an inhibitory effect on natural microflora growth compared to a control washing with deionised water. In the same way the EW treatment used did not affect the surface structure of the leaves after washing and kept the main quality attributes throughout 11 days at 5 °C. The effects of NEW and AEW on by-product formation as well as on processing equipment should be further studied.

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CHAPTER 3

Optimization of acidified sodium chlorite to improve quality of fresh-cut Tatsoi baby leaves

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3.1. INTRODUCTION

Fresh-cut products are highly perishable, due to physiological and microbial factors, and must be prepared for marketing and consumption following strict control procedures. These stringent process controls are essential to assure safety and to delay and reduce overall quality loss. Many of the minimal processing techniques are focused on assuring food safety and high quality products to consumers (Artés *et al.*, 2009). The specific operations involved in fresh-cut preparation can stimulate microbial growth, which may include pathogens harmful to human health. Prevention and sanitation are the most important tools for keeping overall quality and safety of fresh-cut plant commodities. Washing and disinfection is a key step that contributes to effectively reducing microbial load across the supply chain (Suslow, 1997).

Chlorine in various forms has been the most widely used disinfectant in the fresh-cut industry. Chlorination of water is one of the primary elements of a properly managed postharvest sanitation program. Water quality management is considered the most important control point to minimize the transmission of pathogens from infested plant produce, water or debris, to non-infested surfaces such as those mechanically injured during harvesting, transportation, handling or processing, wounds, or the natural plant surface openings (Artés *et al.*, 2009).

Sodium hypochlorite (NaClO) is a potent disinfectant with strong oxidizing properties, being the most commonly used by the fresh-cut industry due to its antimicrobial activity and low cost. However, some problems have been identified related to its use, such us potentially hazardous disinfection-by-products formation, its strong pH dependence, and the potential for gas emission that may affect worker safety (Ölmez and Kretzschmar, 2009). NaClO may partially oxidize food constituents generating unhealthy by-products, such as chloroform ($CHCl_3$), haloacetic acids or other trihalomethanes (THM) that have known or suspected carcinogenic or mutagenic potential effect, with proven toxicity to liver and kidney tissues (Nieuwenhuijsen *et al.*, 2000; Hrudey, 2009). For these reasons, some European countries have forbidden its use in the fresh-cut industry and this may lead to new regulatory restrictions in the near future. Due to the combination of issues previously mentioned, it is imperative to investigate new alternatives which could replace NaClO in the fresh-cut industry.

The antimicrobial activity of ASC is attributed to the oxidative effect of chlorous acid ($HClO_2$), which is derived from the conversion of chlorite ion into its acid form under strong acidic conditions. It is hypothesized that the mode of action of ASC derives from the uncharged $HClO_2$, which is able to penetrate bacterial cell walls and disrupt protein synthesis (Kemp *et al.*, 2000).

Inatsu *et al.* (2005b) evaluated the efficacy of ASC for reducing pathogenic bacteria on lightly fermented Chinese cabbage leaves. Washing inoculated leaves with distilled water reduced the population of *E. coli* O157:H7 by less than 1 log CFU/g,

whereas treating with 0.5 g/L ASC reduced the population by more than 2 log CFU/g. In the same way, Allende *et al.* (2009) found reductions of 3 log CFU/g for *E. coli* O157:H7 populations on fresh-cut cilantro after washing with 1,000 mg/L ASC compared to control. A reduction of viable aerobes of 1 log CFU/g for fresh-cut cantaloupes treated with 1 g/L ASC has been recently reported (Fan *et al.*, 2009).

The use of ASC has been more widely studied as a sanitizer for the meat industry (Castillo *et al.*, 1999; Hajmeer *et al.*, 2004) as compared to the limited research evaluating the effect of ASC microbial and sensory quality on fresh produce, especially leafy vegetables.

The objective of the current work was to elucidate: (1) the decontamination efficacy of ASC on natural leafy green associated microbiota (total aerobic mesophilic and total coliforms); (2) the effectiveness of ASC for controlling inoculated *E. coli*; and (3) the impact of these washing treatments on sensory and keeping-quality of fresh-cut Tatsoi baby leaves, which is an increasingly used component of fresh-cut mixed salads.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

Tatsoi (*Brassica rapa* var. *rosularis*) baby leaves were grown under the Mediterranean climate of the Campo de Cartagena (Murcia, Spain) and mechanically harvested at a commercial development stage. Immediately after harvesting, the leaves were transported by car in about 30 min to the pilot plant of the Technical University of Cartagena (UPCT) where they were air precooled at 5°C. Leaves where stored at this temperature until next morning when minimal processing started.

3.2.2. Bacterial strain and growth conditions

A non pathogenic *E. coli* strain from the Spanish Type Culture Collection (CECT 515) was used in this study. An antibiotic-resistant derivative strain to nalidixic acid (50 mg/L) (Nal^r) was isolated via spontaneous mutation and used to minimized interference with other bacteria and to facilitate the detection and recovery. *E. coli* Nal^r was grown overnight in 50 mL of TSB supplemented with 50 mg/L of nalixidic acid at 37°C. After incubation, cultures were centrifuged at 8,000 g for 5 min to collect the cells and washed twice with 0.1% buffered peptone water (BPW) (Scharlau Chemie S.A., Barcelona, Spain). The final cell pellet was suspended in 0.1% BPW to make an initial cell density (OD_{600}) of approximately 0.7, which corresponds to a concentration of 10^8 UFC/mL. The cell suspension was diluted with deionized water at 10°C to achieve a final concentration of 10^6 UFC/mL. The final concentration was confirmed by plating on Chromocult® coliform agar (Merck, Barcelona, Spain) supplemented with 50 mg/L of nalixicid acid.

3.2.3. Inoculation procedure

Tatsoi leaves were inoculated in a disinfected processing room at 8±1°C by dip inoculation. Leaves were submerged in the inoculum (10^6 CFU/mL) for 5 min and were further removed, drained, spreaded over sterile paper towels and dried in a biosafety cabinet for 1 h at room temperature. Inoculated leaves were stored at 5°C and 90% RH overnight prior to processing.

3.2.4. Sample preparation, treatments and storage conditions

Two different sets of Tatsoi baby leaves, for control and inoculation experiments, were minimally processed separately in a disinfected room at 8°C. First at all, leaves with defects such as yellowing, decay, cuts and bruising were carefully discarded. The raw material was prewashed for 1 min with tap water at 5°C to remove traces of soil and organic matter. The following chemical washing treatments were prepared: ASC at 100, 300 and 500 mg/L by acidification of sodium chlorite (Panreac Quimica S.A.U., Barcelona, Spain) with citric acid to reach a final pH around of 2.8 at 5°C. A standard industrial disinfection treatment with NaClO (100 mg/L, pH 6.5, T = 5°C) was also prepared. As control washing with tap water at 5°C was used. All washing solutions were prepared immediately before application.

The contact times of the different treatments with ASC were 60, 90 and 120 seconds. For the standard chlorinated water and the control treatment the contact time was 120 seconds. All disinfection treatments were followed by a 1 min rinsing step with tap water at 5°C. Then, leaves were manually spin dried to eliminate water excess. An amount of 40 g of leaves was placed in 1,500 mL polypropylene (PP) baskets thermally sealed at the top with a bi-oriented PP (BOPP) film of 40 µm thickness (Plásticos del Segura S.L., Murcia, Spain) to generate a passive modified atmosphere package (MAP). The O_2 and CO_2 transmission rate at 23°C and 0% RH of BOPP was similar with 800 cm^3 /m^2d atm and the water vapour transmission rate at 23°C and 85% RH was 0.7 g /m^2d atm (data provided by the supplier). Three replicates of one basket per treatment and storage temperature and duration (processing day and after 5, 8 and 11 days of shelf life) were prepared and stored in a dark cold room at 5°C and 10°C. The 5°C temperature was selected as the maximum limit recommended and the most commonly used for fresh-cut plant commodities throughout its commercial distribution and retail sale, the 10°C was selected as an abusive temperature during distribution and retail sale.

3.2.5. Gas composition within packages

Gas composition (O_2 and CO_2) within packages was monitored on the processing day and after 5, 8 and 11 days of storage by using a gas-analyzer (PBI Dansensor, CheckPoint, Ringsted, Denmark). In order to avoid modifications in the headspace due to gas sampling, each analyzed package for each evaluation time was opened and used for further analysis. In the current work only results from the samples

subjected to the 120 s contact time are presented. Three replicates were made for each treatment and evaluation period.

3.2.6. Microbial analysis

To determine the microbial growth, standard enumeration methods were used. On each evaluation time three random samples were taken. A 10 g sample of leaves were homogenized in 90 mL of sterile BPW (Scharlau Chemie SA, Barcelona, Spain) for 1 min in a sterile stomacher bag (Model 400 Bags 6141, London, UK) using a masticator (Colwort Stomacher 400 Lab, Seward Medical, London, UK). Tenfold dilution series were prepared in 9 mL of sterile BPW. From non inoculated samples and *E. coli* Na^r from inoculated samples, total aerobic mesophilic bacteria and total coliforms were recovered. The following media and incubation conditions were used: plate count modified agar (Scharlau Chemie, Barcelona, Spain) for mesophilic aerobic bacteria, incubated at 30ºC for 48 h, Chromocult® coliform agar (Merck, Barcelona, Spain) for total coliforms, incubated at 37ºC for 24 h, and Chromocult® coliform agar supplemented with 50 mg/L of nalixicid acid for *E. coli* Na^r, incubated at 37ºC for 24 h. All microbial counts were reported as log colony forming units per gram (log CFU/g). For the non inoculated samples, the presence of *Salmonella* spp., *Listeria monocytogenes* and generic *E. coli* was evaluated according to the European Union legislation for fresh-cut commodities (Regulation EC 1441/2007, 2007). All analyses were made in triplicate.

3.2.7. Sensory evaluation

For the sensory assay non inoculated samples washed 120 s with the different treatments were used. A seven people panel (aged 24-62) trained in sensory quality analyses performed the evaluation. Before running the experiments a consensus was reached between the panellists in order to select those attributes that better describe sensory changes. Sensory quality was evaluated on the processing day and after 5 and 11 days of shelf life at 5ºC and 10ºC. Visual symptoms of dehydration and browning, and off-odours and off-flavours development were scored on a five-point scale of damage incidence and severity (1 = none, 2 = slight, 3 = moderate, limit of usability, 4 = severe and 5 = extreme). Visual appearance and overall quality were evaluated by using a nine-point scale (1 = extremely poor, 5= fair, limit of usability, 9 = excellent) according to Tomás-Callejas *et al.* (2011a).

3.2.8. Contact time/dose optimization assessment

3.2.8.1. Leaves inoculation

The inoculation procedure with *E. coli* Na^r of Tatsoi leaves has been described above. A total of 5 leaves (n=5) were evaluated for each treatment (dose and contact time).

3.2.8.2. Washing treatments

The following chemical treatments were prepared as previously described in 1 L beakers: ASC at 50, 100, 200, 300, 400 and 500 mg/L. To determine the effect of ASC doses on decontamination efficacy, a total of 5 inoculated leaves were each submerged into the sanitizing solution for 5, 15, 30, 60 and 90 s. Then, the leaves were rinsed with deionised water for 10 s in order to eliminate the residual excess sanitizer. This experiment was performed at room temperature (about 22ºC).

3.2.8.3. Bacterial enumeration

Following each treatment, samples were transferred into sterile plastic bag (Whirl-Pak, Nasco, Modesto, California, US) containing sterile BPW (Scharlau Chemie SA, Barcelona, Spain) added in a 1:2 w/v ratio. Samples were then massaged by hand for 1 min. Ten fold dilutions were prepared with BPW. For *E. coli* Na^r recovery, Chromocult® coliform agar (Merck, Barcelona, Spain) supplemented with 50 mg/L of nalixicid acid was used and incubated at 37ºC for 24 h. Results were reported as log reductions of *E. coli*. Five replicates for each dose and contact time were made.

3.2.9. Statistical analysis

The experiment was based on a factorial design: treatments (at three levels of concentration 100, 300 and 500 mg/L) x contact time (at three levels 60, 90 and 120 s) x storage time (four levels 0, 5, 8 and 11 days) at each storage temperature. For the statistical analysis Statistical Analyses System (SAS) 9.2. (SAS Institute, Cary, NC, USA) was used. Replication of each treatment, contact time and storage time combination was repeated in triplicate. Comparison among treatments was carried using the MIXED procedure and Tukey's multiple comparisons for mean separation. A comparison of the ASC treatments during each day of storage against NaClO and water washing treatments was carried using the GLM procedure and Tukey's multiple comparison was utilized for mean separation. For all comparisons, a significant differences were established when $p<0.05$. All data was previously evaluated for normality and homogeneity of variance using the UNIVARIATE procedure function of SAS.

3.3. RESULTS

3.3.1. Gas composition within packages

The gas partial pressure changes of O_2 and CO_2 within packages throughout 11 days at 5ºC and 10ºC are shown in Fig. 1. The initial gas composition was air (20.9 kPa O_2 and 0.03 kPa CO_2). As expected, due to respiratory activity of the leaves and the film permeability, the O_2 levels decreased and the CO_2 increased along the storage time for both storage temperatures. No significant differences ($p<0.05$) were found among treatments at the same storage temperature. Equilibrium gas partial pressures within

packages at 5ºC were reached after 8 days with around 10-12 kPa O_2 and 8-10 kPa CO_2 (balanced with N_2) (Fig. 1A). The same trend was found at 10ºC although higher O_2 consumption and CO_2 emissions occurred in leaves within packages. After 5 days at 10ºC (Fig. 1B), the CO_2 partial pressure was higher than the O_2 for this abusive storage temperature. In this case the equilibrium gas partial pressures within packages were also reached after 8 days with around 1-4 kPa O_2 and 13-15 kPa CO_2.

3.3.2. Sensory evaluation

The average of the scores for sensory evaluation of fresh-cut Tatsoi at 5ºC and 10ºC are shown in Figure 2. After 5 days at 5ºC, Tatsoi baby leaves did not show noticeable symptoms of browning, off-odours nor off-flavours that exceeded the acceptable limit for fresh consumption. However, for all treatments, after 11 days at 5ºC slight dehydration symptoms were recorded. In general, all samples showed a moderate decrease of their overall sensory quality after 11 days at 5ºC, being scored above the limit of usability. Strong differences between storage at 5ºC and 10ºC in visual appearance and overall quality were observed. After 5 days at 10ºC, all treatments except NaClO, were scored below 6 for visual appearance and overall quality and slight dehydration symptoms were observed. After 11 days at 10ºC, none of the treatments were acceptable for consumption based on overall quality and slight to moderate off-flavours were registered for all samples (data not shown).

3.3.3. Microbial analysis

3.3.3.1.Natural microflora

The initial microbial load for total aerobic mesophilic bacteria was 4.39 log CFU/g (Table 1A). Washing for 120 s with NaClO resulted in 1 log reduction of the population compared to the control. Significant differences ($p < 0.05$) among ASC and NaClO treatments compared to the control were found. The antimicrobial effect of ASC against NaClO was similar after washing and no significant differences were registered. After 5 days at 5ºC, the mesophilic population reached 5.74 log CFU/g and remained stable throughout the chilling storage. For NaClO, ASC 300 and ASC 500 lower bacteria populations were registered and no significant differences among them were found. After 5 days at 5ºC, ASC 100 was not as effective as NaClO, ASC 300 and ASC 500 treatments and showed a similar behaviour to the control. The initial antimicrobial effect of the sanitizing solutions did not remain until the end of the chilling storage. The bacterial load was similar for all treatments after 11 days at 5ºC. Regarding the length of the treatment, comparisons within each treatment were carried out. In general, no significant differences between contact times were found.

Total mesophilic counts of fresh-cut Tatsoi leaves stored at 10ºC are shown in Table 1B. As expected, the microbial growth rate was faster than at 5ºC. The bacterial population in the NaClO and the ASC treatments was higher than that in control

samples after 8 days at 10ºC. At the end of shelf life, ASC 500 mesophilic load was above 1 log CFU/g compared to the control. No differences between contact times within each treatment were found.

Changes in total coliforms population as affected by treatment, contact time and storage duration are listed in Table 2. NaClO and ASC were effective in reducing coliforms (> 1 log reductions) populations on fresh-cut Tatsoi compared to the control. For most of sanitizing treatments, total coliforms counts were below the limit of detection (2 log CFU/g) during the MAP storage at 5ºC (Table 2A). Nevertheless, coliforms populations in ASC treated leaves were above than those in control during the storage at 10ºC (Table 2B). Significant differences were registered for all the sanitizing treatments after 11 days compared to the control while no differences between contact times within each treatment to any storage temperature were found.

Generic *E. coli*, *Salmonella* spp., and *Listeria monocytogenes* were not recovered from any of the non-inoculated samples, being safe according the EC legislation (Regulation EC 1441/2007, 2007).

3.3.3.2. Escherichia coli

The populations of *E. coli* on fresh-cut leaves treated with several sanitizing solutions and MAP stored up to 11 days at 5 and 10ºC are listed in Table 3. The *E. coli* population attached to Tatsoi leaves after washing with water (control) was 5.15 log CFU/g. Washing with NaClO reduced the *E. coli* population in 1.17 log units. Treatment with NaClO and ASC 100 resulted in similar reductions in the number of viable *E. coli* and no significant differences were found among those treatments. The antimicrobial effect of ASC at a concentration of 300 and 500 mg/L was more effective than the NaClO treatment in reducing *E. coli*. ASC 500 treatment showed the greatest log reductions of 1.76, 1.56 and 1.88 at 60, 90 and 120 s of contact time respectively compared to the control. No *E. coli* growth during MAP storage at 5ºC for any treatment was found. The effects of the antimicrobial solutions remained throughout shelf life. After 11 days at 5ºC, log reductions ranging from 1.6 to 2.3 for ASC 300 and ASC 500 compared to the control were achieved. No differences between contact times within each treatment were found.

Contrary to the observations at 5ºC, storage at 10ºC resulted in an increase of the *E. coli* population during the MAP storage for all treatments (Table 3B). For control treatment the *E. coli* load reached 6.07 log CFU/g after 11 days at 10ºC. The *E. coli* populations for the remaining treatments were below the control but only ASC 300 (60 s) and ASC 500 showed significant differences. The microbial growth rate along the storage time was faster for the samples treated with chemical solutions than for the control. After 11 days at 10ºC, *E. coli* population increased in 0.92 and 1.78 compared to the initial load for the control and NaClO treatments respectively and above 2 log

units for all ASC treatments. In general, no differences between contact times within each treatment were found.

3.3.4. Contact time/dose optimization assessment

The measured inoculum level for the generic *E. coli* was 6.38 ± 0.02 log CFU/mL (data not shown). The survival of *E. coli* on Tatsoi baby leaves exposed to the different ASC doses and the different contact times of treatments are shown in Fig. 3. After inoculation, on average, 5.35 ± 0.06 log CFU/g of attached *E. coli* was detected.

Washing Tatsoi leaves for 5 s with 50, 100, 200, 300, 400 and 500 mg/L of ASC reduced 0.51, 1.19, 1.07, 1.11, 1.72 and 1.89 log CFU/g of the *E. coli* population respectively. Between ASC 50 and the two highest concentrations (400 and 500 mg/L) significant differences ($p<0.05$) in log reductions were found. The antimicrobial effects of ACS were more noticeable during the first 30 seconds of contact time. In addition, higher doses resulted in higher reductions. Increased time of contact after the first 30 s did not increase the antimicrobial effect of ASC which remained quite stable. After 90 s of contact time, log reductions above 2 for all treatments were recorded. No differences in populations between treatments after 90 s of contact time were found.

3.4. DISCUSSION

Prevention and sanitation are the most important tools for keeping microbial quality and safety of fresh-cut commodities. The limitation of the postharvest washing-disinfection treatment to remove the microorganisms acquired in the field or during handling with chemical sanitizers, as well as the main produce-related foodborne pathogens, is becoming of an important interest (Artés and Allende, 2005; Kim *et al.*, 2009; Ölmez and Temur, 2010). Washing with water physically removes microorganisms from the surfaces of fresh plant produces, but previous reports have shown little decontamination effect on several whole and fresh-cut commodities (Park *et al.*, 2008; Tomás-Callejas *et al.*, 2011b). In fact, washing with water does not guarantee the inactivation of foodborne pathogens. Several issues previously discussed, related with the use of chlorine as sanitizer in the fresh-cut industry have stimulated in finding alternative sanitizers. The use of ASC has been widely study in the poultry and meat industry. Moreover, there is a concern about the use of this chemical in the food industry due to its acidic nature. As far as we know, there are little studies about the corrosive effect of ASC on stainless steel and food processing equipment.

Mesophilic bacteria counts typically range from 3 to 9 log CFU/g for fresh produce and from 3 to 6 log CFU/g for processed produce (Zagory, 1999). In this study, less than 2 log CFU/g reductions of aerobic microflora, coliform population and *E. coli* in fresh-cut Tatsoi leaves compared to water-washed samples was achieved. This result agrees with that reported by Ruíz-Cruz *et al.* (2006), who found reductions by 1.2 – 2 log CFU/g in shredded carrots treated with ASC (100, 250 and 500 μL/L) compared to water-washed and unwashed samples. In the same way, reductions in aerobic

mesophilic bacteria, yeast and moulds by about 2 log CFU/g for fresh-cut cilantro by Allende *et al.* (2009).

Many research studies reported the antimicrobial effect of the sanitizers only after their application. However, the maintenance of the microbiological reduction reached during storage is even more important. Natural epiphytic microflora growth during storage at 10ºC was faster for the disinfected treatments with NaClO and ASC than for the control. A hypothesis for this behaviour is that ASC treatment could cause superficial damages on the leaves, releasing nutrients and promoting microbial growth. Ruíz-Cruz *et al.* (2006) reported that shredded carrots at 5ºC resulted in more electrolyte leakage than lower ASC concentrations and chlorine, probably due to tissue damage caused by ASC. However, further studies should be performed to support this claim. Also, it should be considered that disinfection does not remove the entire associated microflora. Hence, the remaining bacteria might find less nutrient competition which could enhance microbial growth during chilling storage. Thus, López-Velasco *et al.* (2010) found that the remaining bacteria in spinach disinfected with NaClO were able to quickly re-establish populations on the surface compared to water-washed samples during storage at 4ºC and 10ºC.

Reductions in population of inoculated *E. coli* on leafy vegetables as affected by ASC have been reported. Washing Chinese cabbage leaves with distilled water could reduce the population of *E. coli* O157:H7 by approximately 1 log CFU/g, whereas treating with 0.5 g/L ASC at 4ºC could reduce the population by 1.6 log CFU/g (Inatsu *et al.*, 2005a). In the current work, NaClO and ASC showed a bactericidal effect in approximately 1.2 and 1.4 – 1.9 log CFU/g reduction of the *E. coli* population (Table 3) respectively. The antimicrobial efficacy of ASC 100 resulted as effective as NaClO, while ASC 300 and ASC 500 were significantly different (Table 3). *E. coli* populations were maintained during the MAP storage at 5ºC for any treatment. This result agrees to another study where no changes in population were found for Iceberg lettuce stored 14 days at 5ºC (Delaquis *et al.*, 2002). Our results also agree with those reported by López-Velasco *et al.*, (2010), where *E. coli* O157:H7 populations on inoculated spinach were maintained after 15 days at 4ºC, while storage at 10ºC resulted in a small increase.

Regarding the contact time no significant differences within treatments was found. In addition, increasing the length of the treatment does not appear to increase the antimicrobial effect. Many research studies focused on the evaluation of sanitizers on the inactivation of foodborne pathogens in fresh plant produces using abusive contact times. In fact, contact time above 2 min is not realistic at industrial level for washing fresh-cut leafy vegetables. Thus, as previously commented, the effect of ASC on *E. coli* (Fig. 3) seems to be more effective only during the first 30 seconds. Although it was not taken into account for the current study, water quality parameters such as turbidity, organic matter content, oxidation reduction potential, conductivity, and temperature should be consider in order to evaluate the real efficacy of ASC. In addition, organic

matter content in the processed water might interfere with the effectiveness of chemical sanitizers. These factors should be included in further studies.

Gas partial pressures within packages after 8 days at 5ºC were around 10-12 kPa O_2 and 8-10 kPa CO_2. This equilibrium partial pressure can be considered as recommended for fresh-cut baby leaf vegetables during shelf life at 5ºC (Conte *et al.*, 2008; Artés-Hernández *et al.*, 2009). However, for all treatments during shelf-life at the abusive temperature of 10 ºC, high CO_2 concentrations were reached and the O_2 levels were close to the theoretical limit of 0.5 kPa for aerobic respiration (Saltveit, 2003) which implies risks of both fermentation and anaerobic microbial growth.

In summary, low to moderate doses of ASC (100-500 mg/L) compared to the maximum allowed by FDA (1,200 mg/L) showed an initial antimicrobial efficacy on reduction of epiphytic microflora and *E. coli* as effective as NaClO regardless of the contact time on fresh-cut Tatsoi baby leaves. Throughout the 11 days of shelf life sensory quality attributes were well kept at 5ºC but not for the abusive 10ºC. Based on our microbial findings, ASC treatment could damage the superficial tissues of fresh-cut Tatsoi baby leaves and could promote microbiological growth. Therefore, studies on the surface damage of the leaves should be further studied, as well as the effects of ASC on nutritional quality of Tatsoi baby leaves and on the processing equipment.

The application of a sanitizer or a combination of disinfection treatments does not mean a sanitation program. It implies much more effort, from a well designed integrated production, handling and processing to proper distribution chains, keeping appropriate chilling storage temperatures and optimal MAP conditions throughout the entire commercial life. In addition, Good Agricultural Practices, Good Manufacturing Practices and Hazard Analysis and Critical Control Points programs should be implemented and accomplished to minimize the risk of contamination and to assure safety and overall quality to consumers.

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FIGURES AND TABLES

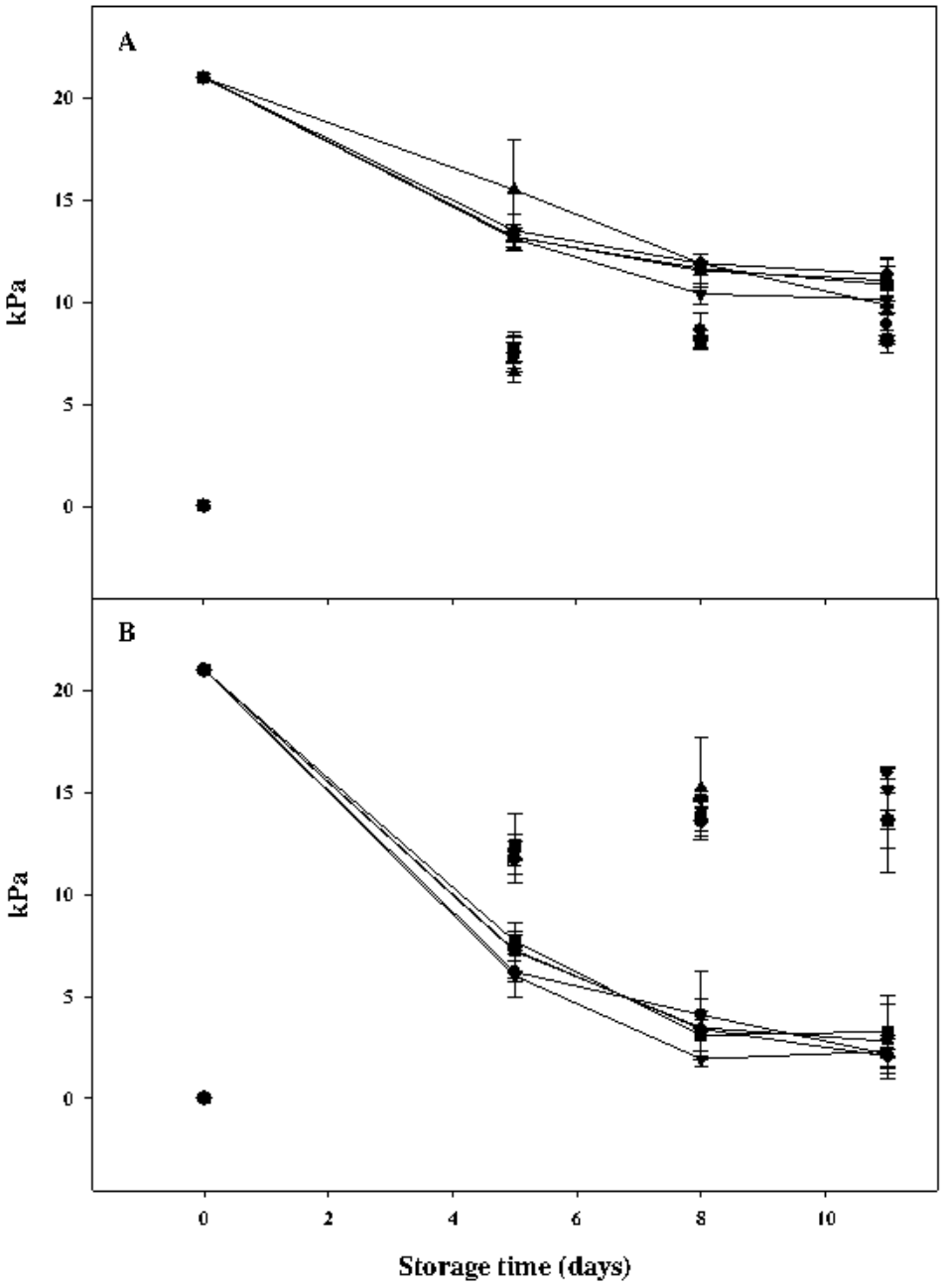
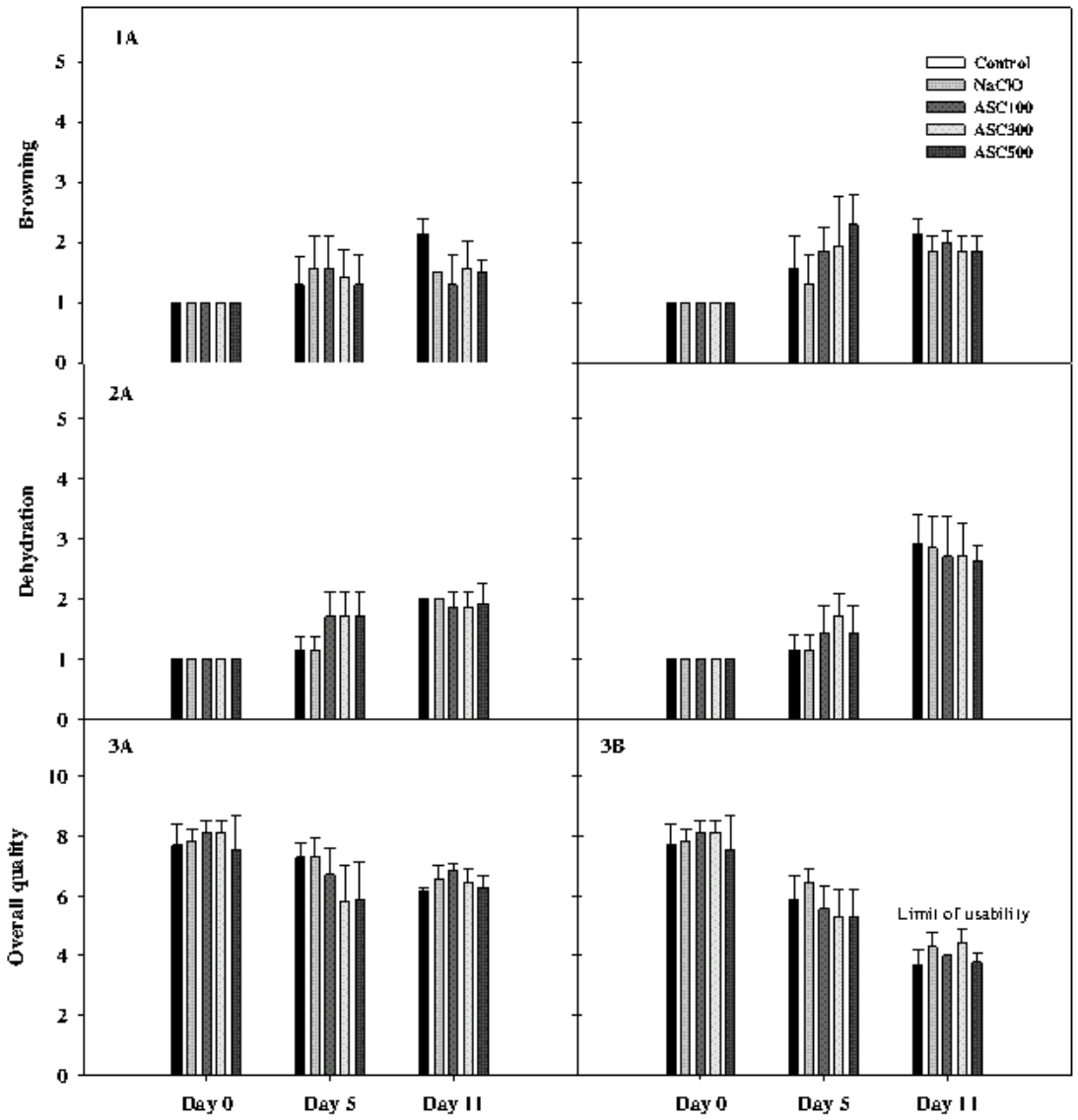


Figure 1. Gas changes within packages of fresh-cut Tatsoi leaves washed under several treatments (●Control; ▼NaClO; ■ ASC 100; ◆ASC 300; ▲ASC 500) and stored under MAP up to 11 days at 5ºC (A) and 10ºC (B). Solid and dashed line represents O_2 and CO_2 concentration respectively. Data represent means of three replicates (n=3 ± SD).



Browning and dehydration scale (1 = none, 2 = slight, 3 = moderate, limit of usability, 4 = severe and 5 = extreme). Overall quality scale (1 = extremely poor, 5= fair, limit of usability, 9 = excellent).

Figure 2. Browning (1), dehydration (2) and overall quality (3) evaluation of fresh-cut Tatsoi baby leaves washed under several ASC treatments and stored under MAP up to 11 days at 5°C (A) and 10°C (B).

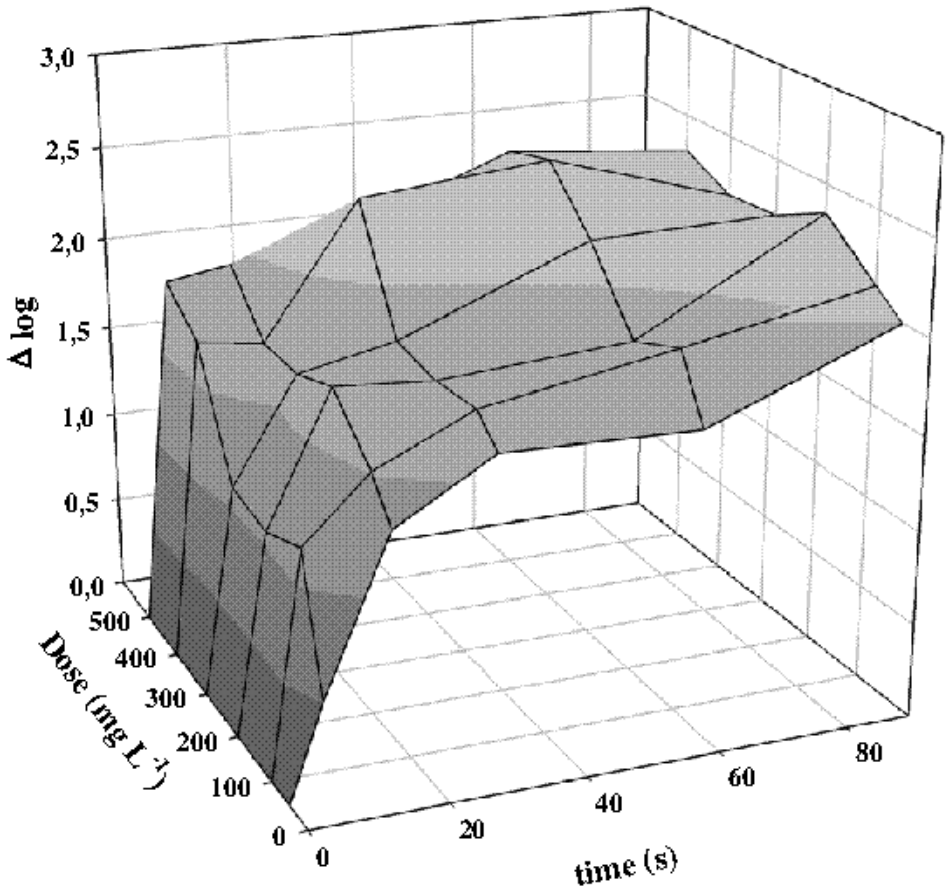


Figure 3. Log reductions of *E. coli* on Tatsoi baby leaves exposed to different ASC concentrations for different contact times (n = 5).

Table 1. Effect of ASC sanitization of fresh-cut Tatsoi leaves on total aerobic mesophilic bacteria stored under MAP at 5°C (A) and 10°C (B) up to 11 days. Data represent means of three replicates (n=3 ± SD).

Table 1A

Treatment	Contact time (s)	Storage time at 5°C (days)			
		Day 0	Day 5	Day 8	Day 11
Water	120	4.39 ± 0.20^{A}	5.74 ± 0.15^{A}	5.53 ± 0.37^{A}	5.81 ± 0.16^{A}
NaClO	120	3.44 ± 0.29^{B}	4.48 ± 0.30^{B}	4.99 ± 1.20^{A}	5.46 ± 0.48^{AB}
ASC 100	60	$3.92 \pm 0.33^{B,1}$	$5.99 \pm 0.19^{A,1}$	$5.05 \pm 0.67^{A,1}$	$4.89 \pm 0.54^{B,1}$
	90	$3.25 \pm 0.81^{B,1}$	$4.26 \pm 0.26^{A,2}$	$5.01 \pm 0.42^{A,1}$	$4.50 \pm 0.24^{B,1}$
	120	$3.39 \pm 0.18^{B,1}$	$5.23 \pm 0.86^{A,1,2}$	$5.86 \pm 0.48^{A,1}$	$5.11 \pm 1.27^{B,1}$
ASC 300	60	$4.00 \pm 0.04^{B,1}$	$4.82 \pm 0.30^{B,1}$	$5.10 \pm 0.20^{A,1}$	$4.65 \pm 0.78^{B,1}$
	90	$3.58 \pm 0.22^{B,1}$	$4.47 \pm 0.20^{B,1}$	$4.83 \pm 0.36^{A,1}$	$4.66 \pm 0.14^{B,1}$
	120	$3.74 \pm 0.21^{B,1}$	$4.22 \pm 0.35^{B,1}$	$4.61 \pm 0.87^{A,1}$	$4.80 \pm 0.42^{B,1}$
ASC 500	60	$3.78 \pm 0.25^{B,1}$	$4.74 \pm 1.11^{B,1}$	$4.64 \pm 0.73^{A,1}$	$5.46 \pm 0.11^{AB,1}$
	90	$3.57 \pm 0.42^{B,1}$	$4.18 \pm 0.29^{B,1}$	$4.97 \pm 1.10^{A,1}$	$4.76 \pm 0.80^{AB,1}$
	120	$3.77 \pm 0.06^{B,1}$	$4.76 \pm 0.09^{B,1}$	$5.02 \pm 0.35^{A,1}$	$5.10 \pm 0.67^{AB,1}$

Table 1B

Treatment	Contact time (s)	Storage time at 10°C (days)			
		Day 0	Day 5	Day 8	Day 11
Water	120	4.39 ± 0.20^{A}	5.78 ± 0.09^{A}	6.65 ± 0.64^{A}	5.77 ± 0.14^{B}
NaClO	120	3.44 ± 0.29^{B}	5.91 ± 0.21^{A}	6.94 ± 0.53^{A}	5.65 ± 0.27^{B}
ASC 100	60	$3.92 \pm 0.33^{B,1}$	$6.02 \pm 0.20^{A,1}$	$7.08 \pm 0.25^{A,1}$	$6.72 \pm 0.16^{B,1}$
	90	$3.25 \pm 0.81^{B,1}$	$5.75 \pm 0.44^{A,1}$	$6.69 \pm 0.30^{A,1}$	$5.86 \pm 0.26^{B,1}$
	120	$3.39 \pm 0.18^{B,1}$	$6.24 \pm 0.04^{A,1}$	$7.43 \pm 0.02^{A,1}$	$7.08 \pm 0.10^{A,1}$
ASC 300	60	$4.00 \pm 0.04^{B,1}$	$6.34 \pm 0.06^{A,1}$	$6.99 \pm 0.26^{A,1}$	$6.69 \pm 0.52^{B,1}$
	90	$3.58 \pm 0.22^{B,1}$	$6.24 \pm 0.39^{A,1}$	$7.04 \pm 0.54^{A,1}$	$6.00 \pm 0.89^{B,1}$
	120	$3.74 \pm 0.21^{B,1}$	$5.95 \pm 0.33^{A,1}$	$7.00 \pm 0.68^{A,1}$	$6.66 \pm 0.36^{B,1}$
ASC 500	60	$3.78 \pm 0.25^{B,1}$	$6.16 \pm 0.05^{A,1}$	$6.76 \pm 0.65^{A,1}$	$6.64 \pm 0.38^{B,1}$
	90	$3.57 \pm 0.42^{B,1}$	$5.75 \pm 0.24^{A,1}$	$6.84 \pm 0.15^{A,1}$	$6.99 \pm 0.12^{A,1}$
	120	$3.77 \pm 0.06^{B,1}$	$5.34 \pm 0.34^{A,1}$	$6.92 \pm 0.20^{A,1}$	$6.54 \pm 0.53^{B,1}$

Different capital letters denote significant difference between the treatments and the two control treatments (water and NaClO) ($p<0.05$) within the same column.

Different number within each column per each treatment and day, denote significant difference in contact time ($p<0.05$).

Table 2. Effect of ASC sanitization of fresh-cut Tatsoi leaves on total coliforms stored under MAP at 5°C (A) and 10°C (B) up to 11 days. Data represent means of three replicates (n=3 ± SD).

Table 2A

Treatment	Contact time (s)	Storage time at 5°C (days)			
		Day 0	Day 5	Day 8	Day 11
Water	120	3.83 ± 0.53 ^{A}	<2.00 ^{B}	4.14 ± 1.86 ^{A}	2.51 ± 0.88 ^{A}
NaClO	120	<2.00 ^{B}	<2.00 ^{B}	2.86 ± 1.49 ^{A}	<2.00 ^{A}
ASC 100	60	2.10 ± 0.17 ^{B,1}	2.49 ± 0.20 ^{A,1}	2.10 ± 0.17 ^{A,1}	<2.00 ^{A,1}
	90	<2.00 ^{B,1}	<2.00 ^{B,1}	<2.00 ^{A,1}	2.39 ± 0.68 ^{A,1}
	120	<2.00 ^{B,1}	2.97 ± 1.05 ^{A,1}	4.54 ± 1.09 ^{A,1}	3.89 ± 0.18 ^{A,1}
ASC 300	60	<2.00 ^{B,1}	2.49 ± 0.20 ^{A,1}	2.16 ± 0.28 ^{A,1}	2.69 ± 1.19 ^{A,1}
	90	<2.00 ^{B,1}	<2.00 ^{B,1}	<2.00 ^{A,1}	2.57 ± 0.99 ^{A,1}
	120	<2.00 ^{B,1}	<2.00 ^{B,1}	<2.00 ^{A,1}	<2.00 ^{A,1}
ASC 500	60	<2.00 ^{B,1}	2.10 ± 0.17 ^{B,1}	<2.00 ^{A,1}	2.88 ± 1.53 ^{A,1}
	90	2.64 ± 1.11 ^{B,1}	<2.00 ^{B,1}	<2.00 ^{A,1}	<2.00 ^{A,1}
	120	<2.00 ^{B,1}	<2.00 ^{B,1}	<2.00 ^{A,1}	2.48 ± 0.84 ^{A,1}

Table 2B

Treatment	Contact time (s)	Storage time at 10°C (days)			
		Day 0	Day 5	Day 8	Day 11
Water	120	3.83 ± 0.53 ^{A}	4.01 ± 0.62 ^{B}	4.12 ± 0.64 ^{B}	3.92 ± 0.88 ^{A}
NaClO	120	<2.00 ^{B}	4.23 ± 0.18 ^{B}	6.39 ± 0.54 ^{A}	5.36 ± 0.43 ^{A}
ASC 100	60	2.10 ± 0.17 ^{B,1}	5.60 ± 0.10 ^{A,1}	6.69 ± 0.08 ^{A,1}	5.46 ± 0.05 ^{A,1}
	90	<2.00 ^{B,1}	2.28 ± 0.49 ^{B,1}	4.58 ± 1.37 ^{B,2}	4.75 ± 1.51 ^{A,1}
	120	<2.00 ^{B,1}	5.60 ± 0.10 ^{A,1}	6.50 ± 0.02 ^{A,1}	6.04 ± 0.09 ^{A,1}
ASC 300	60	<2.00 ^{B,1}	4.80 ± 0.24 ^{A,1}	6.04 ± 0.12 ^{A,1}	5.50 ± 0.55 ^{A,1}
	90	<2.00 ^{B,1}	5.21 ± 0.43 ^{A,1}	5.88 ± 0.26 ^{AB,1}	4.61 ± 0.86 ^{A,1}
	120	<2.00 ^{B,1}	4.98 ± 0.53 ^{A,1}	5.21 ± 0.61 ^{AB,1}	5.21 ± 0.29 ^{A,1}
ASC 500	60	<2.00 ^{B,1}	4.99 ± 0.60 ^{A,1}	5.30 ± 0.22 ^{AB,1}	4.93 ± 0.49 ^{A,1}
	90	2.64 ± 1.11 ^{B,1}	4.59 ± 0.43 ^{A,1}	4.77 ± 0.14 ^{AB,1}	5.95 ± 0.26 ^{A,1}
	120	<2.00 ^{B,1}	4.32 ± 0.92 ^{A,1}	4.67 ± 0.92 ^{AB,1}	3.89 ± 1.26 ^{A,1}

Different capital letters denote significant difference between the treatments and the two control treatments (water and NaClO) ($p<0.05$) within the same column.

Different number within each column per each treatment and day, denote significant difference in contact time ($p<0.05$).

Limit of detection = 2 log CFU/g.

Table 3. Effect of ASC sanitization of fresh-cut Tatsoi leaves on *E. coli* stored under MAP at 5°C (A) and 10°C (B) up to 11 days. Data represent means of three replicates (n=3 ± SD).

Table 3A

Treatment	Contact time (s)	Storage time at 5°C (days)			
		Day 0	Day 5	Day 8	Day 11
Water	120	5.15 ± 0.02^{A}	4.80 ± 0.05^{A}	4.93 ± 0.01^{A}	4.69 ± 0.09^{A}
NaClO	120	3.98 ± 0.13^{B}	3.55 ± 0.10^{B}	3.26 ± 0.13^{B}	3.39 ± 0.08^{B}
ASC 100	60	$3.88 \pm 0.14^{B,1}$	$3.57 \pm 0.12^{B,1}$	$3.42 \pm 0.04^{B,1}$	$3.33 \pm 0.11^{B,1}$
	90	$3.85 \pm 0.08^{B,1}$	$3.44 \pm 0.11^{B,1}$	$3.23 \pm 0.23^{B,1}$	$3.20 \pm 0.32^{B,1}$
	120	$3.86 \pm 0.11^{B,1}$	$3.47 \pm 0.14^{B,1}$	$3.21 \pm 0.22^{B,1}$	$3.25 \pm 0.03^{B,1}$
ASC 300	60	$3.76 \pm 0.13^{B,1}$	$3.18 \pm 0.13^{B,1}$	$2.94 \pm 0.30^{B,1}$	$2.79 \pm 0.10^{B,1}$
	90	$3.53 \pm 0.07^{C,1}$	$3.07 \pm 0.13^{B,1}$	$2.74 \pm 2.24^{C,1}$	$2.78 \pm 0.16^{B,1}$
	120	$3.42 \pm 0.20^{C,2}$	$2.78 \pm 0.16^{C,1}$	$2.75 \pm 0.18^{C,1}$	$2.84 \pm 0.28^{B,1}$
ASC 500	60	$3.39 \pm 0.08^{C,1}$	$3.13 \pm 0.49^{B,1}$	$2.72 \pm 0.22^{C,1}$	$3.10 \pm 0.09^{B,1}$
	90	$3.59 \pm 0.14^{C,1}$	$2.87 \pm 0.15^{C,1}$	$2.84 \pm 0.06^{B,1}$	$2.90 \pm 0.55^{B,1}$
	120	$3.27 \pm 0.09^{C,1}$	$2.80 \pm 0.30^{C,1}$	$2.59 \pm 0.11^{C,1}$	$2.32 \pm 0.55^{C,1}$

Table 3B

Treatment	Contact time (s)	Storage time at 10°C (days)			
		Day 0	Day 5	Day 8	Day 11
Water	120	5.15 ± 0.02^{A}	4.80 ± 0.05^{A}	5.72 ± 0.08^{A}	6.07 ± 0.04^{A}
NaClO	120	3.98 ± 0.13^{B}	3.55 ± 0.10^{AB}	5.48 ± 0.16^{AB}	5.76 ± 0.12^{AB}
ASC 100	60	$3.88 \pm 0.14^{B,1}$	$3.57 \pm 0.12^{A,1}$	$5.38 \pm 0.07^{A,1}$	$5.69 \pm 0.35^{AB,1}$
	90	$3.85 \pm 0.08^{B,1}$	$3.44 \pm 0.11^{B,1}$	$5.14 \pm 0.27^{A,1}$	$5.99 \pm 0.02^{AB,1}$
	120	$3.86 \pm 0.11^{B,1}$	$3.47 \pm 0.14^{A,1}$	$5.18 \pm 0.22^{A,1}$	$5.72 \pm 0.15^{AB,1}$
ASC 300	60	$3.76 \pm 0.13^{B,1}$	$3.18 \pm 0.13^{B,1}$	$4.98 \pm 0.15^{A,1}$	$5.62 \pm 0.23^{AB,1}$
	90	$3.53 \pm 0.07^{C,1}$	$3.07 \pm 0.13^{C,2}$	$4.92 \pm 0.06^{A,1}$	$5.22 \pm 0.17^{C,1}$
	120	$3.42 \pm 0.20^{C,2}$	$2.78 \pm 0.16^{C,1}$	$4.38 \pm 0.14^{C,1}$	$5.40 \pm 0.18^{BC,1}$
ASC 500	60	$3.39 \pm 0.08^{C,1}$	$3.13 \pm 0.49^{C,1}$	$4.87 \pm 0.30^{A,1}$	$5.31 \pm 0.14^{BC,1}$
	90	$3.59 \pm 0.14^{C,1}$	$2.87 \pm 0.15^{C,1}$	$4.69 \pm 0.48^{B,1}$	$4.90 \pm 0.27^{C,1}$
	120	$3.27 \pm 0.09^{C,1}$	$2.80 \pm 0.30^{C,1}$	$4.77 \pm 0.93^{B,1}$	$5.30 \pm 0.18^{BC,1}$

Different capital letters denote significant difference between the treatments and the two control treatments (water and NaClO) ($p<0.05$) within the same column.

Different number within each column per each treatment and day, denote significant difference in contact time ($p<0.05$).

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CHAPTER 4

Chlorine dioxide and chlorine effectiveness to prevent *Escherichia coli* O157:H7 and *Salmonella* cross contamination on fresh-cut Red Chard

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4.1. INTRODUCTION

It is well known that a diet rich in fruit and vegetables can promote health benefits, including the prevention of chronic diseases such as cancer or cardiovascular diseases (Steinmetz and Potter, 1996; Liu, 2003). An increasing deterrent to consumption and realizing these health benefits, fresh produce consumed raw has become widely recognized as a vehicle for transmitting infectious diseases (Leistner and Gould, 2002; Harris *et al.*, 2003). Enteric diseases linked to consumption of fresh produce have dramatically increased in the last several decades (Scallan *et al.*, 2011; Morris, 2011). *Escherichia coli* O157:H7 has been associated with multiple outbreaks linked to the consumption of fresh and fresh-cut leafy vegetables (CDPH, 2004; CDPH, 2005; CDC, 2006). Recent *Salmonella* outbreaks associated to leafy vegetables in Europe and the US have been also reported (Hanning *et al.*, 2009; Raybaudi-Massilia *et al.*, 2009).

The specific operations involved in preparation of fresh-cut vegetables can facilitate attachment and stimulate microbial growth, which includes diverse complexes of spoilage microbiota and bacterial pathogens harmful to human health (Sapers *et al.*, 2006). Therefore, fresh-cut produce must be managed in primary production phases and elaborated for marketing following strict control procedures for reducing overall quality loss and assuring its safety to consumers (Artés *et al.*, 2009).

Immature or baby leafy vegetables are typically consumed raw. Hence, washing and disinfection is a key step that contributes to effectively reducing microbial load across the supply chain (Beuchat, 2000; Artés *et al.*, 2009). Chlorine (sodium hypochlorite –NaClO–) in various approved agricultural and food grade formulations have been the most widely used disinfectant in the fresh-cut industry. However, there is a certain concern about the use of NaClO by the fresh-cut industry due to its low effectiveness in presence of organic matter, and the formation of known or potentially carcinogenic or mutagenic by-products such as chloroform and haloacetic acids (Nieuwenhuijsen *et al.*, 2000; Richardson *et al.*, 2007; Hrudey, 2009; Ölmez and Kretzschmar, 2009).

Chlorine dioxide (ClO_2) has been utilized as a water treatment for several decades but has attracted increased interest over the past ten years as an alternative to NaClO for the fresh and fresh-cut produce industry (Artés *et al.*, 2009; Gómez-López *et al.*, 2009). ClO_2 used as a disinfectant has several accepted or perceived advantages over NaClO, including higher oxidant capacity (Bernarde *et al.*, 1967), effectiveness over a broad range of pH (Parish *et al.*, 2003), lower reactivity with organic matter (Gordon and Rosenblatt, 2005) and high effectiveness at low concentrations (Huang *et al.*, 1997). In addition, ClO_2 and its main by-product, chlorite (ClO_2^-), are classified as non-carcinogenic products (IARC, 1991; EPA, 2000; ATSRD, 2004). However, as with all chemistries used for process water and wastewater disinfection, ClO_2 has disadvantages associated with its use. ClO_2 is a very unstable substance; it is highly explosive as a

concentrated gas (and therefore must be generated on-site). It decomposes readily when exposed to sunlight, as may occur in raw produce washing operations (Suslow, 1997).

Regardless of these practical challenges, the efficacy of ClO_2 in inactivating key foodborne pathogens of concern among different commodities has been reported. Concentrations of 4-5 mg/L were effective to reduce *Salmonella* spp, *Escherichia coli* O157:H7 and *Listeria monocytogenes* inoculated onto cabbage, carrot, lettuce, strawberry and melon (Sy *et al.*, 2005; Mahmoud *et al.*, 2007; Mahmoud and Linton 2008; Keskinen *et al.*, 2009). ClO_2 can also decrease the viability of *Cryptosporidium parvum* oocysts (Peeters *et al.*, 1989).

Washing procedures applied to fresh produce have the potential to reduce contamination from the surface of the product. However, the wash water may also serve as a source of contamination or has great potential to result in cross-contamination within a lot and among sequentially washed lots. Due to economic and environmental factors, reconditioning and recycling of water has been a long employed practice for the industry and it is currently recommended by governmental institutions (USDA, 1999). The effective use of chemical sanitizers during the washing-disinfection step is a critical point for keeping quality and safety of fresh-cut produce.

Despite the wide use of antimicrobial chemistries add to water as processing aides, the survival of food borne pathogens on fresh-cut products and resultant illness and outbreaks remains a reality. The rapid detection of human pathogens at various points from preharvest to finished, packaged product has become a priority for the fresh-cut industry (Gómez *et al.*, 2010). Fresh-cut leafy vegetables are highly perishable, and hold times for pathogen test outcomes, consistent with conventional culture-based methods are unacceptable, even when sufficient large capacity refrigerated storage is available. Conventional methods of detecting pathogens in food samples are time-consuming, taking 3 to 5 days or longer for a confirmed positive result and intrinsic traits of some leafy vegetables can prevent or delay detection with rapid commercial detection kit formats (D'lima and Suslow, 2009). In addition, even a few surviving cells, at numbers below typical limits of detection are still being cause for concern. In the case of *E. coli* O157:H7, for example, evidence from epidemiological studies suggests that the infectious dose may be as few as 10 cells per serving (Jinneman *et al.*, 1995). In recognition of this risk to consumers, research efforts have been focused on the development more rapid, unequivocal and sensitive pathogen detection methods. Several commercially developed and approved or certified rapid methods for the detection of low levels of *E. coli* O157:H7 and *Salmonella* in fresh-cut leafy vegetables are available. The Qualicon BAX® Salmonella system and the Assurance GDS® O157:H7 are PCR-based systems and currently approved by the AOAC as official methods for detection of *Salmonella* and *E. coli* O157:H7 in selected foods (Silbernagle *et al.*, 2003; Feldsini *et al.*, 2005). Despite these advances, the optimal sampling locations for assessing pathogen risk exposure and control efficiencies during fresh-cut processing of salad blends have yet to be fully elucidated.

Baby leafy vegetables have grown in popularity as a base ingredient for fresh-cut mixed salads. Baby Red Chard (*Beta vulgaris* cv. cicla) is one of the most commonly consumed which adds a sweet with slightly bitter flavor undertones to mixed salads.

The objective of the current work was to evaluate process handling cross-contamination potential and pathogen removal of initially low numbers of attached cells of *Salmonella* and *E. coli* O157:H7 during the washing-disinfection, rinsing, and de-watering steps of fresh-cut Red Chard (*Beta vulgaris* cv. cicla). To determine this, a model wash-process system was designed to evaluate efficacy and stability of liquid ClO_2 applied to the water as the disinfectant treatment.

4.2. MATERIALS AND METHODS

4.2.1. Liquid chlorine dioxide stability

A synthetic processing water to simulate commercial processing water conditions was created. To simulate the background oxidative demand of processing water, plant material with adhered soil was taken from University of California (Davis, CA) research farm and submerged in tap water. Dilutions were prepared and adjusted to create synthetic process water with turbidity values of 22 and 160 FAU (Formazin Turbidity Unit ≅ Nephelometric Turbidity Unit). These assays were carried out by adding ClO_2 (Aquapulse Systems Inc., San Luis Obispo, CA, USA) by serial dilutions in 100 mL of synthetic water to reach a final concentration of 3 mg ClO_2/L confirmed by using a colorimeter as described below. Changes in oxidation reduction potential (ORP) and residual ClO_2 concentration (mg/L) during a 2 min interval at two temperatures (10 and 25°C) and two water turbidities (22 and 160 FAU) were monitored. All physicochemical parameters were determined using standard protocols. Specifications of the instruments used are as follows: ORP-sensor (Thermo Fisher Scientific Inc., Waltham, MA, USA) for ORP; portable colorimeter for turbidity (DR/850, Hach Company, Loveland, CO, USA) and ClO_2 residual (Pocket Colorimeter™ II, Hach Company, Loveland, CO, USA). The samples of 160 FAU water were filtered by using a 0.45 µm filter before measuring the ClO_2 residual to avoid interference among suspended solids and the ClO_2 colorimeter. All analyses were made in triplicate.

4.2.2. Plant material

Red Chard (*Beta vulgaris* cv. cicla) leaves were grown under certified organic management in Salinas Valley (California, US) and hand harvested at a commercial development stage as immature or 'baby' leaves. Immediately after harvesting, the leaves were placed in pre-cooled, insulated chests (coolers) and transported to the Mann Lab (University of California, Davis) where they were held at 5°C until further processing within 18 hours.

4.2.3. Bacterial cultures and inoculum preparation

Attenuated strains of *E. coli* O157:H7 (PTVS155) and *S. enterica* sv. Typhimurium (PTVS177) were used in this study (Table 1). A derivative strain resistant to rifampicin (80 mg/L) was isolated for each strain via selection of spontaneous mutants and used to minimize interference with other bacteria during quantitative and qualitative detection and determinative confirmation tests (Beuchat *et al.*, 2001). Both strains were grown separately overnight in 9 mL of Tryptic Soy Broth (TSB) (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin at 37°C. After incubation, cultures were centrifuged at 4,000 x *g* for 10 min. The pellet was re-suspended and washed twice with Butterfield's phosphate buffer (Whatman Inc., Piscataway, NJ, USA). The final cell pellet was suspended in Butterfield's phosphate buffer to obtain an initial cell density (OD_{600}) of approximately 0.7, which corresponds to a concentration of 10^8 CFU/mL. The final concentration was confirmed by serial dilution plating on Tryptic Soy Agar (TSA) (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin (TSA-rif). All experimentation was conducted in the Suslow Biosafety Level 2 Laboratory of the L. Mann Postharvest Laboratory at University of California, Davis.

4.2.4. Inoculation procedure

Red Chard leaves were submerged in phosphate buffer (pH 7.0) containing 10^5 CFU/mL for 1 min and were subsequently dewatered and dried overnight at 15°C and 95% RH prior to processing to allow for presumptive cell attachment on the plant phyllosphere. Inoculated leaves were marked in the distal tip of the petiole with a colored tape for differentiation from non-inoculated leaves combined with treated leaves during processing.

4.2.5. Sample preparation and processing

Two sets of different experiments with Red Chard baby leaves, separately for *Salmonella* and *E. coli* O157:H7, were subjected to simulated minimally processing at room temperature (nominally 20°C). In each experiment as a preparative step, non-inoculated leaves with defects such as yellowing, decay, harvest and transport damage, and bruising were discarded. Twenty inoculated and marked Red Chard leaves were mixed with 300 g (roughly 120 leaves) of non-inoculated leaves from the same lot and processed as a unit. Within each replicated experiment and each specific treatment variation, inoculated material represented the 3-5% of the total weight. Two different model washing systems were evaluated as follows:

A. Prewashing (1 min, spray–shower with municipal tap water), Disinfection Wash (1min, sanitizing solution –aerated batch agitation wash), Rinsing (30 s, spray-shower with municipal tap water) and Dewatering (30 s – manual centrifugation with 10 L foodservice spin dryer).

B. Prewashing (1 min, immmersion with municipal tap water), Disinfection Wash (1 min, sanitizing solution – immersion), Rinsing (30 s, spray-shower with municipal tap water) and Dewatering (30 s – manual centrifugation with 10 L foodservice spin dryer).

Prewashing, washing-disinfection and rinsing were performed in different tanks using a ratio of 5 L water/300 g product. The aerated agitation bath was created by passing compressed air through a 1.5 x 35 cm-long aquarium airstone. Two chemical disinfectant solutions: ClO_2 (3 mg/L) (Aquapulse Systems Inc. San Luis Obispo, CA, USA) and NaClO (6%) (The Clorox Company, Oakland, CA) (25 mg/L, adjusted to pH 6.5 with citric acid), were prepared immediately before application and added in the washing-disinfection tank. After processing, inoculated and non-inoculated leaves were collected, divided by type and analyzed separately. The experiment was repeated twice for each strain.

4.2.6. Bacterial detection, recovery and enumeration

4.2.6.1. Water

Three random water samples of 20 mL were collected from each washing step (prewashing, washing, rinsing and centrifugation) to evaluate the potential transfer of pathogens from inoculated leaves to the processing water. Samples were immediately neutralized using sodium thiosulphate ($Na_2S_2O_3$) (1N). The amount of $Na_2S_2O_3$ used was previously tested in the laboratory under anticipated experimental conditions and was added in excess to ensure the complete ClO_2/NaClO neutralization. Recovery was done by 0.45 μm hydrophobic grid filtration using the Neogen ISO-GRID system (Neogen Corporation, Lansing, MI, USA). Membranes containing any viable captured bacterial cells were transferred to TSA-rif and incubated at 37°C for 24 h. TSA-rif plates were also supplemented with 1 g/L of sodium pyruvate {$C_3H_3NaO_3$;(TSARP)} during preparation to facilitate resuscitation of sub-lethally injured cells (Knudsen *et al.*, 2001). Following incubation, the grid squares containing presumptive positive colonies were counted to yield CFU/10mL.

4.2.6.2. Red Chard

Inoculated and marked leaves (n=20) were collected after processing and placed in 5 sterile bags (Whirl-Pak™, Nasco, Modesto, California, US) containing 4 leaves each and 0.1% sterile buffered peptone water (BPW) (BD Diagnostics, Sparks, MD, USA) added in a 1:2 w/v ratio. Samples were then vigorously massaged by hand for 1 min. Ten-fold dilutions were prepared with BPW and plated on TSARP for both *E. coli* O157:H7 and *S. enterica* recovery. Plates were incubated at 37°C for 24 h, counted and results reported as log CFU/g. Five replicates for each strain; sanitizing type and washing system were performed.

Three replicates (n=3) of 25 g of non-inoculated leaves were placed in sterile bags containing 50 mL of buffer supplemented with 80 mg/L of rifampicin to evaluate the potential transference of pathogens from inoculated material to non-inoculated leaves. BPW and mEHEC (Biocontrol Systems Inc., Bellevue, WA, USA) were used as broth enrichment media for *Salmonella* and *E. coli* O157:H7, respectively. Samples were homogenized for 30 s at medium speed in a Pulsifier (Microgen Bioproducts Ltd., Surrey, UK) and direct plated on TSARP plates. Then, enrichment cultures were further supplemented with BPW or mEHEC, supplemented with rifampicin as above, to reach a final volume of 225 mL. Broth enrichments were incubated for 24 h at 37°C without shaking and subsequently plated on TSARP, as above, and also small aliquots were removed for PCR-based detection as described below. Results from plates were reported as log CFU/g.

4.2.6.3. Inoculated bacterial isolate detection by PCR

Pathogen detection kits were used to evaluate the potential cross-contamination by *Salmonella* and *E. coli* O157:H7 from inoculated to non-inoculated plant material during processing, anticipated to be below the quantitative limit of enumeration. BAX® Salmonella (Dupont/Qualicon, Wilmington, DE) and GDS® O157:H7 (BioControl, Bellevue, WA) were used in this study as rapid detection kits. BAX® Salmonella is based on real-time detection of SYBR intercalation into double-stranded DNA during PCR amplification. Standard detection time includes 8 h of non-selective enrichment plus 3 h for processing and detection. For these experiments, a selective enrichment by the addition of rifampicin was used to enhance the recovery of applied isolates. Assurance GDS® O157:H7 is a probe-based real time PCR which is combined with an immunomagnetic separation step prior to processing, amplification, and detection. Time to detection includes 8 h of semi-selective enrichment plus 75 min for amplification and detection. The specific technical directions provided with each kit were followed carefully with the exception of the use of rifampicin supplementation. Enriched samples were incubated according to provider specifications and results were used to determine presence/absence of the target pathogen. All analyses were made in triplicate.

4.2.7. Statistical analysis

The data was analyzed by using Statistical Analyses System (SAS) 9.2. (SAS Institute, Cary, NC, USA). Each treatment was repeated in triplicate. Comparison among treatments was carried using the GLM procedure and Tukey's multiple comparison was utilized for mean separation. For all comparisons, a significant difference was established when $p<0.05$. All data were previously evaluated for normality and homogeneity of variance using the UNIVARIATE procedure function of SAS.

4.3. RESULTS

4.3.1. Liquid chlorine dioxide stability

Changes in ORP and residual ClO_2 as affected by water temperature and turbidity are shown in (Fig. 1). Residual ClO_2 concentrations were monitored after its addition during a 2 min interval. The impact of turbidity was more pronounced than the effects of water temperature in ORP and residual ClO_2 values. From an initial ClO_2 concentration of 3 mg/L, residuals quickly decreased to 1 mg/L within the first 20 s when added to 160 FAU turbidity water regardless of the water temperature and subsequently remained quite constant during the remaining 2 min (Fig. 1A). A turbidity value of 22 FAU at a water temperature of 10ºC was less influential in affect on reducing the initial ClO_2 concentration during the monitored time. However, a slight decrease of residual ClO_2 for 25ºC water temperature within 2 min was observed. In general, lower reductions of residual ClO_2 were observed at 10ºC than at 25ºC as well as at 22 FAU than at 160 FAU. A ClO_2 concentration of 3 mg/L was associated with ORP values greater than 650 mV for water turbidity of 22 FAU at 10 or 25ºC. However, when water turbidity was 160 FAU, ORP values decreased throughout the time and dropped to values less than 500 mV within 10 s. For the ClO_2 concentration tested, ORP values were not significantly different irrespective of water temperature tested for each turbidity condition (Fig. 1B).

4.3.2. Pathogen inactivation on inoculated leaves

Inoculated leaves represented 3-5 % of total weight of minimally processed Red Chard. After washing the combined leaf mass, inoculated and marked leaves were selectively removed and analyzed separately. The antimicrobial effects of ClO_2 and NaClO on *E. coli* O157:H7 and *S. enterica* are presented in Table 2.

The initial population of *E. coli* O157:H7 on inoculated leaves before processing was 3.51 ± 0.13 log CFU/g. For the washing type A, log reductions after processing of 0.70 reaching a final load of 2.80 log CFU/g for both sanitizing solutions were achieved. Washing type B was apparently more effective for NaClO compared to washing type A and a reduction of 0.85 log CFU/g just after washing was observed. In general, no significant differences ($p<0.05$) among washing types and sanitizers for *E. coli* O157:H7 were found.

Greater log reductions for *S. enterica* as compared to *E. coli* O157:H7 after processing were reached. The initial *Salmonella* load was 4.27 log CFU/g. Significant differences in log reductions after washing among sanitizers within the same washing system were found. Washing with NaClO resulted in 1.50 and 1.12 log CFU/g reductions of *Salmonella* for the washing types A and B respectively. Reductions of 0.88 and 1.53 log CFU/g after washing with ClO_2 for the model systems A and B respectively were achieved.

4.3.3. Pathogen cross-contamination from inoculated leaves to processing water and to non-inoculated leaves

The potential cross-contamination from inoculated leaves to non-inoculated ones via processing water was studied (Table 3). After processing the Red Chard leaves, no *bona fide* colonies on selective media were recovered and, therefore, levels of the applied pathogen surrogate isolates were below the limit of quantitative detection (1.4 log CFU/g) regardless of the sanitizer and the washing model type used. Neutralized water samples from prewashing, washing, rinsing and the dewatering step were processed by using a sterile hydrophobic grid membrane to evaluate the potential risk of *E. coli* O157:H7 transference from inoculated leaves to the processing water (Table 3). No colonies in the processing water from prewashing, washing and rinsing steps for any sanitizer and washing type were detected. However, small populations ranging 0.3 – 0.5 CFU/10mL of *E. coli* O157:H7 were recovered from effluent discharge water collected during the centrifugation de-watering step.

In this study PCR-based rapid detection kits were used to evaluate the presence of the pathogens below the quantitative limit of detection in non-inoculated Red Chard leaves (Table 3). For the washing type A, the probe-based GDS system provided molecular evidence of the presence of *E. coli* O157:H7 after washing with NaClO in 1 out of 3 samples, whereas no positives for ClO_2 treated washes were registered. Positives observed within model system A represented 6% of the total samples for both sanitizers. However, 50% of the analyzed samples were positive for washing system B. Regarding the obtained results, none of the sanitizers in the conditions assayed, could completely prevent the cross-contamination to non-inoculated leaves within the limits of the model system design. Although it is not part of the official protocol, the selective enrichments used for the GDS procedure were direct plated on CHROMagar O157^{TM} (CHROMagar, Paris, France) to confirm the GDS results and verify that PCR detection included viable target cells.

As observed for *E. coli* O157:H7, no colonies of *Salmonella* were recovered from non-inoculated leaves regardless of the sanitizer and the washing model type used after processing. However, transference of viable *Salmonella* from inoculated leaves to the processing water was detected. Water from the centrifugation step was found to harbor significant populations of the target bacteria. Populations of *Salmonella* in the NaClO treated samples were lower than in comparison to ClO_2, independent of the washing system. The *Salmonella* recovery in the centrifuge effluent water was 55 and 83 CFU/10 mL for NaClO and 139 and 114 CFU/10 mL for ClO_2 for the washing types A and B, respectively. No significant differences among washing systems for each sanitizer were found.

The evaluation of the presence of *Salmonella* in non-inoculated leaves below the quantitative limit of detection was carried out by using the BAX® *Salmonella* system. None of the sanitizers in the conditions assayed were able to prevent cross-

contamination to non-inoculated leaves and all samples were determined to be positive by the BAX assay. The selective enrichments used for the procedure were also plated out on XLT4 and found to confirm the PCR assay results.

4.4. DISCUSSION

Fresh-cut produce is commonly consumed raw and disinfection in a washing step constitutes a minimum practical means of decontamination for quality and safety. Washing with water, particularly with added mechanical action, physically removes microorganisms from the surfaces of plant products, and the addition of disinfectants can help to improve the efficacy of this processing step. However, several reports have shown that the level of decontamination is not high enough to ensure safety of fresh-cut commodities (Ruíz-Cruz *et al.*, 2007; Park *et al.*, 2008; Tomás-Callejas *et al.*, 2011). Consequently improvements in the application of chemical sanitizers to the washing water are a crucial point to prevent cross-contamination and maximize inactivation of attached human pathogens.

The population of *E. coli* O157:H7 and *Salmonella* inoculated onto Red Chard leaves was determined after the chemical treatment. In this study, 0.7 and 0.8-1.5 log reductions of *E. coli* O157:H7 and *Salmonella*, respectively, after processing with 3 mg/L ClO_2 were observed. This result is consistent with that previously reported by López-Gálvez *et al.* (2010) where generic *E. coli* reductions of 0.8 log CFU/g in fresh-cut lettuce treated with 3 mg/L ClO_2 were detected. However, inconsistent results have been reported depending on the commodity, initial inoculum level, ClO_2 concentration, contact times, washing conditions and recovery procedures (Singh *et al.*, 2002, 2003; Lee and Bake, 2008; Pao *et al.*, 2007, 2009). Washing iceberg lettuce for 2 min with TriNova (20, 100 and 200 mg/L of ion ClO_2^-) resulted in reductions of *E. coli* O157:H7 of 0.96, 1.05 and 1.13 log CFU/g respectively (Keskinen *et al.*, 2009). Increasing the ClO_2 concentration does not seem to result in greater reductions of the initial pathogen population. In the same manner, Kim *et al.* (2009) reported log reductions of 1.66 and 1.54 log CFU/g of *E. coli* O157:H7 and *S. enterica* ser. Typhimurium in inoculated broccoli sprouts after washing with 50 mg/L ClO_2 for 5 min. However, the current residual ClO_2 concentration in water approved by the U.S. Food and Drug Administration must not exceed 3 mg/L for fresh cut produce (21 CFR 173.300; 2007).

The inoculated plant material represented only 3-5 % of total weight when mixing with non-inoculated Red Chard leaves. Low levels of inoculum and the inoculated/non-inoculated plant material ratio was selected to mimic real conditions of contamination in the industrial processing plants. Plant material might arrive from the production fields with sporadic levels of contamination within and among lots received by the processor. Lots may include product harvested from different production fields and even different growers many miles apart. Non quantitative evidence of cross-contamination of non-inoculated leaves by *E. coli* O157:H7 was detected after plating on selective culture media (LOD 1.52 log CFU/g). Based on the Assurance GDS results,

33 % of the total samples were positive for *E. coli* O157:H7, and only ClO_2 for the washing type A was able to completely prevent cross-contamination. Prior reports demonstrated that once cross-contamination occurs during produce washing, further washing with disinfectant solutions including ClO_2, is unable to control the contamination (López-Galvez *et al.*, 2010). While no *Salmonella* colonies were recovered on selective media from non-inoculated Red Chard, after detection by PCR-BAX all samples were positive regardless of the washing and sanitizer type used. Pao *et al.* (2007) studied the effect of ClO_2 for preventing *Salmonella* cross-contamination through contaminated water to non-inoculated tomatoes and determined that 5 mg ClO_2/L effectively prevented cross-contamination.

It is known that water quality can affect the efficiency of chemical sanitizers. Although the interaction between ClO_2 and organic matter does not produce toxic by-products, as occurs with NaClO, ClO_2 is able to oxidize a large fraction of natural organic matter (Swietlik and Sikorsa, 2004), reducing its availability in inactivating target microbes. Cross-contamination with *E. coli* between inoculated and non-inoculated fresh-cut escarole washed with different water quality was influenced by microbial and organic load present in re-circulating water (Allende *et al.*, 2008). In this study, ClO_2 stability test results showed that ORP values were affected by water turbidity. It has been established that ORP values >650 mV are required to effectively kill human pathogens in the limited contact times typically present during produce washing processes (Suslow, 2004). The ORP often correlates well with the antimicrobial potential of the water and it is associated with the concentration of the oxidant, though not in a direct linear fashion under all conditions (Robbs *et al.*, 1995). Previous studies showed that residual ClO_2 in hot water was lower than in cold water, presumptively related with faster solubilization of ClO_2 (Zhang *et al.*, 2008).

Though further study is warranted, our results suggest that special attention to water from a centrifugation-dewatering step should be taken into account as a control point in minimal processing of fruits and vegetables. Large populations of *Salmonella* were recovered from centrifugation discharge effluent water whereas no colonies were detected from water in contact with inoculated leaves in earlier washing unit operations. This result confirms the ability of the centrifugal force applied during the de-watering step to remove at least a proportion of more firmly attached cells with the unbound water from the leaf surface. This represents a potential risk of cross-contamination transference to product and equipment at the step immediately prior to packaging. These results further suggest that the centrifugation effluent water could be used as a potential sample point to evaluate lot contamination and cross-contamination in the processing chain by low levels of pathogens, as were used in this study, undetectable by conventional sampling methods. In this way, a rapid molecular protocol for evaluating aerobic bacterial load on fresh-cut lettuce by using centrifugation water as an alternative to the food product itself has been recently developed (Gómez *et al.*, 2010). In the same way, Bhagwat (2004) proposed to use vegetable rinse-water as a broader lot screening strategy for detecting *Salmonella* by real time PCR.

In summary, selected levels of ClO_2 and NaClO used in this experiment were unable to fully disinfect the applied pathogen surrogates from inoculated leaves regardless of the washing type. While ClO_2 substantially prevented *E. coli* O157:H7 cross contamination, of the isolate used in this study, it was not effective for the *Salmonella* isolate. Due to the certainty of generating aerosols and other means of handling contamination, only individual isolates of the available attenuated pathogen surrogates could be used to represent possible outcomes with pathogenic forms, as would be typical in more contained lab studies of wash processing.

The outcomes of this research provide further evidence that the application of chemical sanitizers during the washing-disinfection step does not guarantee the inactivation of pathogens and elimination of cross-contamination concerns. Water quality management parameters should be designed thoughtfully and implemented with great attention to detail to optimize safety and overall quality for consumers.

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TABLES AND FIGURES

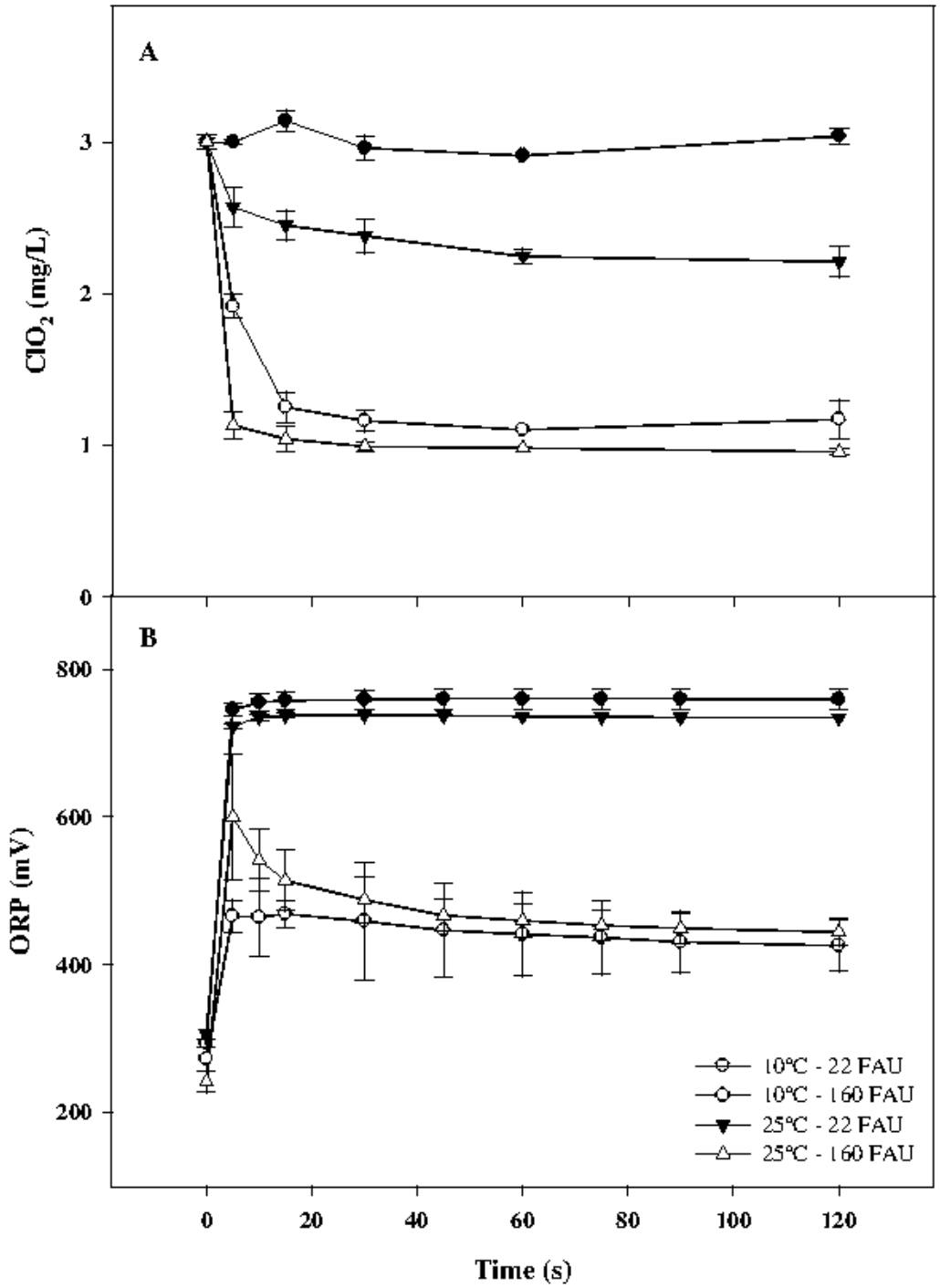

Figure 1. Effect of water temperature (°C) and turbidity (FAU) on the residual ClO_2 concentration (mg/L) and ORP (mV) within 2 min of contact time.

Table 1. Characteristics of the *Escherichia coli* O157:H7 and the *S. enterica* sv. Typhimurium strains used in the study.

Strain	Source of information
PTVS 155	*E. coli* O157:H7. ATCC # 700728, does not possess *stx1* and *stx2* genes (BSL-1). Transformed rifampicin resistant.
PTVS 177	*S. enterica sev.* Typhimurium. χ3895 phage type13a Plasmid: $pSeV2^{+}$. Transformed rifampic resistant. From parent strain PTVS 150, originally made available by R. Curtis; Biodesign Institute, Arizona State University.

Table 2. *E. coli* O157:H7 and *S. enterica* sv. Typhimurium populations (log CFU/g) on inoculated Red Chard leaves as affected by sanitizer and washing types before and after processing.

Escherichia coli O157:H7

		Microbial population (log CFU/g)		
Washing type	Sanitizer	Before processing	After processing	Log reduction
A	NaClO	3.51 ± 0.13	2.80 ± 0.12	0.71
	ClO_2	3.51 ± 0.13	2.79 ± 0.23	0.72
B	NaClO	3.51 ± 0.13	2.66 ± 0.19	0.85*
	ClO_2	3.51 ± 0.13	2.85 ± 0.08	0.66

S. enterica sv. Typhimurium

		Microbial population (log CFU/g)		
Washing type	Sanitizer	Before processing	After processing	Log reduction
A	NaClO	4.27 ± 0.10	2.77 ± 0.12	1.50*
	ClO_2	4.27 ± 0.10	3.39 ± 0.23	0.88*
B	NaClO	4.27 ± 0.10	3.15 ± 0.09	1.12*
	ClO_2	4.27 ± 0.10	2.74 ± 0.05	1.53*

* Denotes significant difference ($p<0.05$) in log reduction within the same row

Table 3. Transference of *E. coli* O157:H7 and *S. enterica* sv. Typhimurium from inoculated Red Chard leaves to processing water as affected by sanitizer and washing types.

Escherichia coli O157:H7				*S. enterica* sv. Typhimurium			
Washing type	Sanitizer	Washing step	CFU/10mL	Washing type	Sanitizer	Washing step	CFU/10mL
A	NaClO	Prewashing	ND[a]	A	NaClO	Prewashing	ND
		Washing	ND			Washing	ND
		Rinsed	ND			Rinsed	ND
		Centrifuged	0.3			Centrifuged	55
	ClO_2	Prewashing	ND		ClO_2	Prewashing	ND
		Washing	ND			Washing	ND
		Rinsed	ND			Rinsed	5
		Centrifuged	0.8			Centrifuged	139
B	NaClO	Prewashing	ND	B	NaClO	Prewashing	ND
		Washing	ND			Washing	ND
		Rinsed	ND			Rinsed	2
		Centrifuged	ND			Centrifuged	83
	ClO_2	Prewashing	0.5		ClO_2	Prewashing	2
		Washing	ND			Washing	ND
		Rinsed	ND			Rinsed	ND
		Centrifuged	0.5			Centrifuged	114

[a]ND: Not detected

Table 4. Cross-contamination of *E. coli* O157:H7 and *S. enterica* sv. Typhimurium from inoculated material to non inoculated Red Chard leaves as affected by sanitizer and washing types above and below the limit of quantitative detection.

Escherichia coli O157:H7				
Washing type	Sanitizer	Microbial counts (log CFU/g)[z]	GDS O157[b]	BAX Salmonella[c]
A	NaClO	ND[a]	1/3	-
	ClO_2	ND	0/3	-
B	NaClO	ND	1/3	-
	ClO_2	ND	2/3	-
S. enterica sv. Typhimurium				
A	NaClO	ND	-	3/3
	ClO_2	ND	-	3/3
B	NaClO	ND	-	3/3
	ClO_2	ND	-	3/3

[z] Limit of quantitative detection LOD = 1.52 log CFU/g.
[a]ND: Not detected
[b]BioControl Assurance GDS E. coli O157 PCR system
[c]Qualicon BAX Salmonella PCR system

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CHAPTER 5

Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions

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5.1. INTRODUCTION

Enteric diseases linked to consumption of fresh produce have dramatically increased in the last several decades (CDC, 2011; Morris, 2011). *Escherichia coli* O157:H7 has been associated in multiple outbreaks linked to the consumption of whole produce and fresh-cut leafy vegetables (CDPH, 2004; CDPH, 2005; CDC, 2006). Diverse opportunities for primary contamination and cross-contamination during preharvest phases and postharvest handling are recognized, including fecal contamination by animals or transmission by insects, use of untreated manure, application of contaminated irrigation or foliar contact water, flood water carrying human waste, and direct human hand contact (Beuchat, 1996; Suslow et al., 2003; Brandl, 2006). Prevention and sanitation become the most important tools for keeping microbial quality and safety of fresh-cut commodities. In response to repeated foodborne outbreaks associated with the consumption of fresh vegetables, the U.S. Food and Drug Administration (FDA) released its "Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables," (FDA, 2008) which identified concerns and provided recommendations for food safety practices that are intended to minimize the microbiological hazards associated with fresh and fresh-cut plant products. Early in 2011, the FDA Food Safety Modernization Act was signed into law and has prompted the produce industry to anticipate minimum federal standards for production of fruits and vegetables based on known safety risks (FDA, 2011).

Consumption of leafy vegetables contaminated with *E. coli* O157:H7 poses an important risk for humans, as epidemiological studies have shown that the infectious dose may be as low as 10 cells (Jinneman et al., 1995). Infection with *E. coli* O157:H7 can cause many forms and severity of illness or morbidity, ranging from rapidly resolving diarrhea to severe hemorrhagic colitis and life-threatening hemolytic uremic syndrome, particularly in infants and elderly people (Delaquis et al., 2007). Plant-based foods had not been traditionally recognized as a host for life-threatening enteric pathogens until multiple factors converged to elevate both the incidence of produce-related outbreaks and the recognition of their role in overall foodborne illness (Warriner and Namvar, 2010).

Surface contamination of the edible tissues of leafy vegetables is often held to be due to pathogen transference from soil or water. Survival of *E. coli* O157:H7 on plant surfaces is variable and controlled by numerous factors including: nutrient availability, competition with indigenous microflora, UV radiation, and relative humidity (Brandl, 2006). Unlike many true epiphytes, *E. coli* O157:H7 does not appear to produce enzymes that degrade plant cell walls and the fate of this enteric pathogen upon arrival in the phyllosphere is not fully understood (Teplitski et al., 2009). A study conducted by Islam et al. (2004) demonstrated that *E. coli* O157:H7 persisted for 154 to 217 days in soils amended with contaminated composts and was detected on lettuce and parsley for up to 77 and 177 days, respectively, after seedlings were planted. In a greenhouse experiment, when *E. coli* O157:H7 was sprayed inoculated (10^4 CFU/mL) on butterhead

lettuce, it was detected after selective enrichment up to 30 days post-inoculation. However, a reduction of the dose level (10^2 CFU/mL) resulted in the absence of detection within 17 days post-inoculation (Salomon et al., 2003). Recently, Moyne et al. (2011) reported that *E. coli* O157:H7 spray-inoculated in a lettuce field was detected up to 28-35 days post-inoculation. Scientific based data modeling the postharvest survival of this enteric pathogen in the phyllosphere of leafy vegetables after processing and retail distribution is abundant but often contradictory (Sapers et al., 2005).

Asian baby leaf vegetables have recently grown in popularity as a base ingredient for fresh-cut mixed salads. Among those Tatsoi (*Brassica rapa* cv. rosularis), Mizuna (*Brassica rapa* cv. japonica) and baby Red Chard (*Beta vulgaris* cv. cicla) are the most commonly consumed. The specific crop cycles for these vegetables vary between 25-75 days depending on the environmental conditions and desired stage of maturity. The specific operations involved in fresh-cut preparation include combined light-handling methods such as washing, trimming or cutting, disinfecting, and packaging at chilled temperatures. Unitization is generally under polymeric films able to develop optimum modified atmosphere packaging (MAP) conditions (Artés and Allende, 2005). Baby leafy vegetables are frequently consumed raw, thus washing and disinfection are a key steps that contribute to effectively reduce microbial load across the supply chain (Suslow, 1997; Allende et al., 2004). Specific postharvest processing operations such as cutting or shredding can damage the plant tissues, rendering them more prone produce to support the survival and growth of pathogenic bacteria responsible for human illness. Postharvest handling practices can influence the fate of *E. coli*, as indicators of enteric contamination, and *E. coli* O157:H7 during processing and could increase the likelihood of cross-contamination (Delaquis et al., 2007; Luo et al., 2011). It has been demonstrated that plant lesions can promote the rapid multiplication of *E. coli* O157:H7 over a short period of time on lettuce during postharvest phases (Brandl, 2008). The application of chemical sanitizers during the washing step constitutes the available and a practical means for achieving some gains in decontamination of leafy vegetables and is one of the primary elements of a properly managed postharvest sanitation program. However, this step does not guarantee the total inactivation of human pathogens (Artés et al., 2009).

The purpose of the current study was to assess the fate of *E. coli* and *E. coli* O157:H7 in diverse baby leafy vegetables from preharvest to postharvest minimal processing, packaging and chilled storage conditions in a model system. Genotyping of applied generic *E. coli* strains to evaluate their comparative survival in the phyllosphere from production throughout processing is also reported.

5.2. MATERIALS AND METHODS

5.2.1. Bacterial strains and inoculum preparation

Two different cocktails of generic *E. coli* (TVS 353, TVS 354 and TVS 355) and *E. coli* O157:H7 (PTVS 154 and PTVS 155) were used (Table 1). Generic *E. coli* strains were isolated from surface irrigation water (TVS 353), Romaine lettuce (TVS 354) and sandy-loam soil (TVS 355) samples from the Central Coast near Salinas, California. Each isolate has a distinct DNA-fingerprint by repetitive extragenic palindromic PCR (Fig. 2). *E. coli* O157:H7 strains used in this study are non-toxigenic isolates (lacking *stx*1 and *stx*2) ATCC 700728 and ATCC 43888, classified as Biosafety Level-1 (BSL-1) and BSL-2, respectively. The use of both *E. coli* O157:H7 strains in greenhouse is currently approved by the Office of Environmental Health and Safety (EH&S) of University of California, Davis. An antibiotic-resistant derivative strain for tolerance to rifampicin (80 mg/L) was isolated via spontaneous mutation and used to minimize interference with other bacteria and to facilitate the detection and recovery for each strain (Beuchat et al., 2001). Both generic *E. coli* and *E. coli* O157:H7 strains were separately grown in 9 mL of tryptic soy broth (TSB) (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin at 37°C for 18h. After incubation, cultures were centrifuged at 4,000 rpm for 10 min. The pellet was re-suspended and washed twice with Butterfield's phosphate buffer (Whatman Inc., Piscataway, NJ, USA). The final cell pellet was suspended in Butterfield's phosphate buffer to achieve a target initial cell density (OD_{600}) of approximately 0.7, which corresponds to a concentration of 10^8 CFU/mL and subsequently diluted to achieve a final concentration of 10^6 CFU/mL. The final concentration was confirmed by plating on Tryptic Soy Agar (TSA) (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin (TSA-rif).

5.2.2. Baby leafy greens cultivation

Seeds of Mizuna (*Brassica rapa* var. japonica), Tatsoi (*Brassica rapa* var. rosularis) and Red Chard (*Beta vulgaris* var. cycla) were supplied by Synergene Seed & Technology (Salinas, CA, USA). Each leafy vegetable was individually grown in aeration container pots of 18 cm in diameter and 10 cm in depth (Smart Pot, High Caliper Growing – Root Control, Inc., Oklahoma City, OK, USA) containing UC mix (33% peat, 25% sand%, 42% fir back). Common commercial plant densities of 900 seeds/m^2 for Red Chard and 1000 seeds/m^2 for Mizuna and Tatsoi were established. Pots were randomly distributed across a greenhouse bench. Greenhouse conditions ranged from 18 to 21°C and from 65 to 75% of relative humidity (RH). Plants were watered daily and fertilized as needed with 50% Hoagland's Solution following standard practices in a research greenhouse of the University of California, Davis. Two development stages of the baby leafy greens were defined for experimental sampling as 3 and 4 weeks old post-planting plants.

5.2.3. Greenhouse trials and inoculation procedure

Two trials were conducted during summer and fall 2010. A factorial combination of the following conditions was evaluated: irrigation type (overhead and drip irrigation), leaf age at inoculation time (3 and 4 weeks post-planting), and inoculum type (generic *E. coli* and *E. coli* O157:H7). Each grouping treatment consisted of 3 pots of each leafy vegetable (n=9), resulting in 54 pots per trial. Each pot was considered as a replication within the same grouping treatment. For the fall trial, only drip irrigation was used.

Hand-spray bottles were used to inoculate the 3 and 4 weeks old plants. The bottles were previously calibrated to release 4 mL of the 10^6 CFU/mL inoculum solution in two single manual pumps of the spray-trigger per pot. The inoculation was always performed between 8 to 8:30 am to prevent acute heat stress. The final bacterial load achieved in each pot was determined to be approximately log 4.2 CFU/cm^2. The final concentration was confirmed by plating on TSA-rif.

Inoculated 3 and 4 weeks old plants were harvested at commercial baby leaf stage after reaching 31 and 38 days post-emergence, respectively, and were subjected to further postharvest operations.

5.2.4. Postharvest operations

After harvest, each grouping treatment was processed separately at room temperature. Leaves with defects such as yellowing, decay, cuts and bruising were carefully discarded. The raw material was washed for 90 s with tap water containing 3 mg/L ClO_2, adjusted from a liquid concentrate, as the disinfectant agent and followed by a 1 min of rinsing with tap water. Then, the leaves were manually spin-dried to eliminate excess water. An amount of 20 g of leaves were placed in 23 x 15 cm commercial salad bags and stored in a cold room without light at 5°C for up to 7 days. The 5°C temperature was selected as the maximum limit recommended for short –term storage and distribution and the most commonly used for fresh-cut plant commodities throughout its commercial retail sale. Three replicates for each grouping treatment were prepared. All the postharvest experimentation was conducted in the Suslow Biosafety Level 2 Laboratory of the L. Mann Postharvest Laboratory at University of California, Davis.

5.2.5. Plant sampling, bacterial recovery and detection

Recovery and detection of generic *E. coli* and attenuated *E. coli* O157:H7 was conducted after 2, 7 and 10 days post-inoculation, following disinfection (AW), and at 7 days of chilling storage (AS). To collect samples, baby leaves were removed by cutting immediately above the soil level with sterile scissors. Plant samples were transferred into sterile plastic bags (Whirl-Pak, Nasco, Modesto, California, US) containing sterile TSB supplemented with 80 mg/L of rifampicin in a 1:2 w/v ratio. Samples were then

massaged by hand for 1 min and 100 µL of bacterial suspension was plated on TSA-rif and incubated at 37ºC for 24 h. TSA-rif plates were also supplemented with 1 g/L of sodium pyruvate {$C_3H_3NaO_3$;(TSARP)} during preparation to facilitate resuscitation of sub-lethally injured cells (Knudsen et al., 2001). Due to the different availability of plant material during the greenhouse development, and in order to homogenize the sample size, an amount of 5 to 15 g of sample was taken for the 2, 7 and 10 days post-inoculation time points and 20 g following disinfection and storage. Results from plate counts were reported as log CFU/g. All analyses were made in triplicates.

Samples with bacterial populations below the limit of detection by direct enumeration, were enriched at a 1:2 w/v ratio using TSB supplemented with 80 mg/L of rifampicin and incubated at 37°C for 18h. For confirmation of generic *E. coli* enrichment was plated onto Chrom-ECC agar (Chrom Agar, Paris, France) supplemented with 80 mg/L of rifampicin and incubated at 37°C. Typical blue colonies were considered a positive result. For detection of *E. coli* O157:H7, amplification of *rfbE* was done using probe based real-time PCR. Amplicons were generated using forward primer *rfbE*-F (5'-GATGCCAATGTACTCGGAAAAAT-3'), reverse primer *rfbE*-R (5'-CCACGCCAACCAAGATCCT-3') and *rfbE* probe (NEDCAAAAGCACCCTATAGCTMGBNFQ). Each 20 µL reaction contained 10 µL of a 2X Taqman® Gene expression master mix (Applied Biosystems Inc., Foster City, CA, USA), 0.5 µM of forward and reverse primers, 2.5 pmol of probe targeting *rfbE* (Applied Biosystems Inc., Foster City, CA, USA) and 2 µL of enrichment that was previously boiled for 95°C for 10 min. Amplification was carried in a thermocycler (7300 Real Time PCR System, Applied Biosystems Inc., Foster City, CA, USA) with a protocol consisting in one cycle of 50°C for 5 min, one cycle of denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 30 seconds and annealing at 60°C for 1 min. Amplification greater or equal to the Ct value of an standard containing 1 copy of *rfbE* was classified as positive.

5.2.6. Repetitive extragenic palindromic PCR (REP-PCR)

Genotyping of individual generic *E. coli* contained in the cocktail was by amplification of repetitive extragenic palindromic (REP) elements to evaluate the distribution of the strains in the phyllosphere of each leafy vegetable from preharvest to postharvest conditions. Ten percent of the total *E. coli* colonies recovered thorough the entire experiments were isolated and used for this assay. Isolated colonies were purified by streaking them on TSA and incubating at 37°C for 24 hours, repeated twice. Purified colonies were re-suspended on Butterfield's phosphate buffer (Whatman Inc., Piscataway, NJ, USA) to yield an OD_{600} of 0.4-0.5. The cell suspension was subsequently centrifuged at 13,000 g for 1 min and resuspended in 200 µL 1X TE buffer. Template DNA was prepared by heating at 95°C for 10 min. Amplification was done with primer REP1R-I (5'-IIIICGICGICATCIGGC-3') and REP2-I: (5'-ICGICTTATCIGGCCTAC-3') (Versalovic et al. 1991). Each 25 µL reaction contained 5 µL 5X colorless GoTaq® buffer (Promega Corp. Madison, WI, USA) supplemented

with 62.5 mmol of MgCl (Invitrogen Corp. Carlsbad, CA, USA), 0.2mM of each dNTP (Applied Biosystems Inc., Foster City, CA, USA), 0.5 µM of REP1R-I and REP2-I primers 1% of DSMO and 1.25 U of GoTaq® (Promega Corp. Madison, WI, USA), 7.75 µL H_2O and 1.5 µL of template DNA. Amplicons were generated in thermocycler (GeneAmp®, PCR Syatem 2700, Applied Biosystems Inc., Foster City, CA, USA) with an initial denaturation (4 min, 94°C) followed by 30 cycles of denaturation (94°C, 1 min), annealing (40°C, 1.5 min) and extension (65°C, 8 min), followed by a single final extension (65°C, 16 min). Amplicons were separated on 1:2 (w/v) agarose for electrophoresis during 2.5 hours at 120 mV. Isolates showing identical band patterns were grouped manually.

5.2.7. Statistical analysis

Statistical analysis was carried with Statistical Analysis Software V. 9.2 (SAS Institute Cary NC) using a factorial design. To assess significant difference among treatments during the time, the MIXED procedure was utilized and Tukey pair comparison was applied for mean separation. A p-value lower than 0.05 was utilized to establish significant difference among treatments. Data was previously analyzed for normality and homogeneity of variance using the UNIVARIATE procedure, function of SAS.

5.3. RESULTS

5.3.1. Environmental conditions during vegetable production

Weekly average temperature, RH and hours of daylight at the greenhouse for both summer and fall trials are shown in Table 2. During the summer trial, the average temperature ranged from 20 to 22°C and the RH around 69-74% during the 5 weeks of experimental baby leaf production. Similar conditions of temperature and RH where recorded during the fall trial; temperature varied from 20 to 21°C and RH from 65 to 72%. Although temperature and RH were similar during both consecutive growing seasons, differences in daylight hours were recorded and light spectrum and quality were correspondingly lower. Average daylight during summer trial ranged from 14.1 to 13.1 h while for the fall trail daylight ranged from 11.7 to 10.6 h, which represents a difference of 3 hours of daylight exposure. Supplemental light was not used.

5.3.2. Survival of generic *E. coli*

A cocktail of three generic strains of *E. coli* were spray-inoculated onto 3 week old or 4 week old Mizuna, Tatsoi and Red Chard leaves. The initial inoculum level was about 4.2 log CFU/cm^2. In general, applied *E. coli* was detected at all the sample points from production to storage regardless of the season, leaf age at inoculation time and irrigation type for all three baby leafy greens evaluated.

For the summer trial, *E. coli* counts 2 days post-inoculation were 2.45 and1.87 log CFU/g for sprinkle and drip irrigation, respectively, for the 3 week-old Mizuna

leaves, while *E. coli* populations on 4 week-old Mizuna leaves, were below the quantitative limit of detection (1.43 log CFU/g) (Table 3). According to the analysis of variance, the leaf age had no effect on the bacterial population ($p>0.05$) during the crop growing for each irrigation type. After 7 and 10 days post inoculation, all the samples were below the limit of detection (LOD) but were detected after selective enrichment. The wash disinfection of Mizuna leaves with ClO_2 was unable to fully inactivate the inoculated bacteria as evidenced by enrichment-detection of *E. coli* survival after processing and storage up to 7 days at 5°C. During the fall trial (Table 5A), *E. coli* populations on the 3 and 4 week-old Mizuna leaves after 2 days post-inoculation were 2.51 and 3.47 log CFU/g respectively. In this trial, the effect of the leaf age on the survival of *E. coli* was significant ($p<0.05$) only at 2 days post-inoculation. *E. coli* populations dropped by approximately 0.75 and 1.66 log CFU/g after 10 days for the 3 and 4 week-old Mizuna leaves, respectively. In contrast with the summer trial, *E. coli* was recovered by direct-plating on selective culture media and the colony counts were above the LOD up to 10 days post-inoculation. Washing harvested material with 3 mg/L of ClO_2 resulted in a reduction of *E. coli* population below the LOD. Similarly to the summer trial, the target bacteria were recovered from all samples after commercial chilling storage of 7 days at 5°C.

In the summer trial for Tatsoi leaves (Table 3), the effect of the leaf age on the survival of *E. coli* was not significant. The *E. coli* populations after 2 days were 3.22 and 3.50 log CFU/g for the 3 week-old leaves subjected to sprinkle and drip irrigation, respectively, and 2.72 and 3.05 log CFU/g for the 4 week-old leaves. The populations remained unchanged during the growing period regardless the irrigation type and dropped by 0.7 – 1 log CFU/g after the wash disinfection with ClO_2 for the 4 week-old leaves. In general, with the exception of the 3 week-old leaves under drip irrigation, *E. coli* population from all the sample points were above of the quantitative LOD. As expected, *E. coli* was still recovered after storage at 5°C for 7 days. A similar trend for the trial performed in fall was observed. *E. coli* population declined in approximately 1.5 log CFU/g after 10 days compared to the population recovered from 2 days post-inoculation. In contrast with the summer trial, the reduction observed for each leaf age was significantly different. After the cold storage, survival of target bacteria was only confirmed by selective enrichment.

In contrast with the previous results, the effect leaf age was significant on the survival of *E. coli* on Red Chard leaves after 2 days post-inoculation for both summer and fall trials. No difference between irrigation types was observed. The number of culturable bacteria declined rapidly from the initial date of inoculation until harvesting and subsequent postharvest washing for the 3 week-old Red Chard leaves in the summer trial. However, the *E. coli* population remained constant from day 2 up to the period of chilling storage and no significant differences ($p>0.05$) were recorded. The same behavior was recorded for *E. coli* population during the fall trial.

5.3.3. Survival of *E. coli* O157:H7

Population dynamics of *E. coli* O157:H7 in the phyllosphere of Mizuna, Tatsoi and Red Chard baby leaves are shown in Tables 4 and 5B for summer and fall trials, respectively. In general, the effect of the irrigation type and leaf age on the survival of *E. coli* O157:H7 was not significant for all leafy green types and trials. For all 3 week-old mini-greens, average *E. coli* O157:H7 population was in the range of 1.8 – 2.4 log CFU/g after two days post-inoculation. However, bacteria were not recovered by direct plating after 2 days post-inoculation from any sampling point. The evidence of *E. coli* O157:H7 survival throughout the simulated processing chain and refrigerated storage conditions was confirmed exclusively by qRT-PCR. Decrease of *E. coli* O157:H7 populations were detected after 2 days post-inoculation for the 4 week-old baby leaves during the summer trial, however populations tended to remain constant from day 7 and during storage. Processing operations, including a washing disinfection step with 3 mg/L of ClO_2, were unable to efficiently remove and/or inactivate the attached *E. coli* O157:H7 from the plant surfaces. The bacteria populations before and after the washing-disinfection operation were not significantly different for all the leafy green.

Seasonal effect for both *E. coli* O157:H7 and generic *E. coli* was evaluated. Significant effect of the growing season was determined for the *E. coli* O157:H7 for the three leafy green varieties, in contrast to the effect of season on generic *E. coli* populations which was found to be only significant for Mizuna but not for Tatsoi or Red Chard.

5.3.4. Distribution of *E. coli* strains in the leafy greens phyllosphere

A cocktail of three different *E. coli* strains (TVS 353, TVS 354 and TVS 355) which were originally isolated from irrigation water, Romaine lettuce leaves, and sandy loam soil cultivated with Romaine lettuce in the Salinas Region (California, USA), was used in this study. REP-PCR, as a genotypic identification method, was utilized in the current study to differentiate the three different strains, which have a different DNA-fingerprint (Fig. 2), and evaluate their relative abundance and distribution in the phyllosphere of the leafy vegetables.

During the summer trial, TVS 353 (water isolate) represented more than the 50% of the culturable bacteria for Mizuna and Red Chard while TVS 355 (soil isolate) population ranged from 20 to 33% for all the leafy green types. TVS 354 (plant) was the least abundant strain with a relative abundance of 9, 16, and 27 % for Tatsoi, Mizuna and Red Chard, respectively, was determined (Fig. 1A).

Similar results were observed for the fall trial where TVS 353 was the most persistent strain. On average, a relative abundance of 56, 46 and 42% of TVS 353 compared to the total populations of applied *E. coli* corresponding to Mizuna, Red

Chard and Tatsoi. TVS 355 represented 33 and 35% of the total for Red Chard and Mizuna while it represented 40% for Tatsoi leaves (Fig 1B).

In general, *E. coli* strains could be ranked according to their persistence in the phyllosphere of the assayed leafy greens as follows: TVS 354 (plant) < TVS 355 (soil) < TVS 353 (water), being the *E. coli* isolated from environmental plant samples the least persistent under the conditions of this study.

5.4. DISCUSSION

This study provides an assessment of indicator/surrogate strains and pathogen behavior under model conditions for the production of baby Mizuna, Tatsoi and Red Chard and including postharvest minimal processing and chilling storage, mimicking commercial conditions. A mixture of three strains of generic *E. coli* and a mixture of two avirulent strains of *E. coli* O157:H7, safe surrogates of the pathogen, were used in this study.

In general, a rapid decline of generic *E. coli* and *E. coli* O157:H7 population for all the leafy vegetables regardless the leaf age and the irrigation type for both seasonal trials was observed. Our results agree with previous findings reported by Wood et al. (2010) for population dynamics of *E. coli* inoculated in growing spinach. In that field study, *E. coli* population was reduced by 3-5 logs after 72 h and culturable bacteria were recovered only up to 6 days post inoculation. Moyne et al. (2011) evaluated the survival of *E. coli* O157:H7 (ATCC 700728) on lettuce plants using a relative low inoculum level (5 log CFU/mL) in a summer field setting experiment. After 2 days post inoculation no culturable *E. coli* O157:H7 were recovered but the target bacteria were detected by selective enrichment up to 14 days post inoculation. A recent study conducted by Erickson et al. (2010), *E. coli* O157:H7 artificially inoculated in a spinach and lettuce field-grown spinach and lettuce at 10^4 CFU/mL via irrigation water was not recovered after 7 days post-inoculation and it could be only detected if the level of the inoculum applied was higher than 10^6 CFU/mL. In contrast with this behavior, *E. coli* O157:H7 was detected up to 77 and 177 days in lettuce and parsley respectively (Islam et al., 2004). Other studies have reported the influence of the irrigation method (Salomon et al., 2002) on the transmission and persistence of the pathogen as well as the leaf age (Brandl and Admunson, 2008) as a risk factor of contamination with *E. coli* O157:H7. In the current study, the leaf age at the time of inoculation as well as the irrigation method used did not have a significant effect on the survival of *E. coli* and *E. coli* O157:H7 during growing phases in a greenhouse.

Solar ultraviolet (UV) radiation and desiccation have been identified as important factors influencing pathogen survival in the phylloplane (Heaton and Jones, 2008). UV radiation contributes to inactivate phyllosphere bacteria and foodborne pathogens of produce (Jacobs and Sundin, 2001, Allende and Artés, 2003). Experiments under greenhouse conditions provide an effective formula to protect the crops from

natural winds and dryness. However, our results did not differ from previous studies, performed under field conditions (Erickson et al., 2010; Wood et al., 2010 Moyne et al., 2011). The decline rate of the survival populations for the fall season was slower than for the summer season, likely result of the differences the duration of daylight exposure among seasons, potentially attributed to longer exposure to solar radiation and a consequence of desiccation at the leaf-air boundary, causing additional stress to bacterial cells on the plant surface.

In the current study we inoculated the leafy vegetables using a cocktail of *E. coli* or *E. coli* O157:H7 strains. This study presents the distribution of the three environmental isolates of generic *E. coli* strains in the phyllosphere of Mizuna, Tatsoi and Red Chard. A heterogeneous distribution of the isolates was observed (Fig. 1). In general, a different relative abundance for each strain was observed, with isolate TVS 353 (water isolate) the most tolerant or best adapted. This corroborates that the use of a mixture of different strains provides a better approach to a real world scenario compared to the use of a single strain.

The minimal processing of the Mizuna, Tatsoi and Red Chard baby leaves included a disinfection step with 3 mg/L of ClO_2 as a disinfection agent. In general, reductions of the population of generic and pathogenic *E. coli* less than 1 log unit for all treatments and leafy green types were achieved. In most cases, these reductions were not significant and ClO_2 was not able to fully disinfect the inoculated leaves. Similar findings have been reported for other minimally processing leafy vegetables such as spinach (Lee and Baek, 2008) and lettuce (Keskinen et al., 2009). Results of this study suggest that the sanitizer was not able to reach the bacteria during the washing step, probably due to their location on the leaves or association within aggregates (Lindow and Brandl, 2003). In fact, bacteria on plant surfaces tend to concentrate where there are more binding and protected sites (Parish et al., 2003), with preferential occurring at the base of trichomes, on the outer rim of stomata, and in cell grooves along veins (Monier and Lindow, 2004). A study of the bacterial distribution on lettuce surface treated with NaClO by scanning electron microscopy showed that the most of the bacteria were located either around or infiltrating stomata or as groups of clustered cells (López-Gálvez et al., 2010). These findings mentioned above suggest that the location of bacteria in sheltered sites could protect them from chemical sanitizers.

After the minimal processing of Mizuna, Tatsoi and Red Chard, they were stored at 5°C which is one of the most commonly used temperatures for minimal processed vegetables throughout its commercial distribution and retail sale. By the end of the simulated shelf-life at 5°C, evidence of survival of *E. coli* and *E. coli* O157:H7 was confirmed by both culture and enrichment or RT-PCR methods for all minimally processed products. In general, the population remained constant or a limited decrease during the cold storage was achieved. Similar results have been reported by other authors. Oliveira et al. (2010) studied the survival and growth of *E. coli* O157:H7 inoculated onto shredded lettuce packaged under MAP at commercial cold storage

conditions. After 10 days at 5°C populations of *E. coli* O157:H7 decreased approximately in 1 log unit. This minimal effect on survival of *E. coli* O157:H7 is similar to other studies with leafy vegetables where no changes in population or small decrease in iceberg lettuce (Delaquis et al., 2002) and spinach (López-Velasco et al., 2010) stored at 5°C and 4°C respectively were found.

The fate of *E. coli* and *E. coli* O157:H7 during production, harvest, processing and storage of Mizuna, Tatsoi and Red Chard baby leaves is reported in the current study. The ability to survive during production and after disinfection and storage of low levels of *E. coli* in the assayed mini greens has been demonstrated. However, field-based trials under realistic conditions to understand the fate of the pathogen should be further studied. In summary, this research work provides useful data to develop an adequate science-base risk assessment during the production and minimal processing of these crops.

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FIGURES AND TABLES

Table 1. Characteristics of the *Escherichia coli* strains used in the study

Strain	Source of information
TVS 353	*E. coli* W778, selected rifampicin resistant. Isolated from irrigation water (Salinas Region, CA, USA).
TVS 354	*E. coli* P149, selected rifampicin resistant. Isolated from lettuce leaves (Salinas Region, CA, USA).
TVS 355	*E. coli* S19, selected rifampicin resistant. Isolated from sandy loam soil (Salinas Region, CA, USA).
PTVS 154	*E. coli* O157:H7. ATCC # 43888, does not possess *stx1* and *stx2* genes (BSL-2). Selected rifampicin resistant.
PTVS 155	*E. coli* O157:H7. ATCC # 700728, non toxigenic (BSL-1). Selected rifampicin resistant

Table 2. Climatic data summary for each trial. Values are the average weekly temperature, relative humidity and daylight hours

	Summer 2010			Fall 2010		
	T (°C)	RH (%)	Daylight hours	T (°C)	RH (%)	Daylight hours
Week 1	21.3	72.5	14.1	20.8	71.6	11.7
Week 2	21.0	73.4	13.8	19.8	69.4	11.4
Week 3	20.1	72.8	13.6	20.2	68.4	11.2
Week 4	21.8	69.4	13.3	19.2	71.8	10.9
Week 5	21.1	71.4	13.1	19.1	65.6	10.6

Table 3. Persistence of *E. coli* inoculated on 3 weeks old (A) and 4 weeks old (B) Mizuna, Tatsoi and Red Chard baby leaves from production to processing and storage (summer 2010)

A		Time after inoculation[a]				
Crop	Irrigation type	2	7	10	AW	AS
Mizuna	Sprikle	2.45_{A}	(3/3)[b]	1.59_{A}	1.79_{A}	1.52_{A}
	Drip	1.87_{A}	(3/3)	(3/3)	(2/3)	(2/3)
Tatsoi	Sprikle	3.22_{A}	3.14_{A}	$*3.49_{A}$	3.37_{A}	2.34_{A}
	Drip	3.50_{A}	(3/3)	(3/3)	(3/3)	(3/3)
Red	Sprikle	$*2.91_{A}$	(3/3)	(3/3)	$*2.33_{AB}$	2.10_{AB}
Chard	Drip	2.62_{B}	(3/3)	$*2.92_{A}$	1.94_{B}	(3/3)
B		Time after inoculation[a]				
Crop	Irrigation type	2	7	10	AW	AS
Mizuna	Sprikle	(3/3)	(3/3)	(3/3)	(2/3)	(2/3)
	Drip	1.52_{A}	(3/3)	(2/3)	(3/3)	(3/3)
Tatsoi	Sprikle	2.72_{AB}	(3/3)	2.97_{A}	2.28_{AB}	2.12_{AB}
	Drip	3.05_{A}	2.31_{A}	2.86_{A}	1.87_{A}	1.90_{A}
Red	Sprikle	3.11_{A}	2.95_{A}	3.05_{A}	3.21_{A}	2.04_{A}
Chard	Drip	3.39_{A}	2.96_{A}	3.38_{A}	2.94_{A}	2.86_{A}

[a] Levels of *E. coli* are expressed as log CFU/g (n=3).

[b] Ratio denotes the number of replications tested positive for *E. coli* by selective enrichment in TSB+rif^{80} and confirmed by surface plating on CHROMECC+rif^{80} per total. Limit of detection is 1.43 log CFU/g.

Different capital letters denote significant difference ($p<0.05$) within the same row.

* Denotes significant difference ($p<0.05$) between the irrigation type for the same crop and time point.

Table 4. Persistence of *E. coli* O157:H7 inoculated on 3 (A) and 4 (B) weeks old Mizuna, Tatsoi and Red Chard baby leaves from production to processing and storage (summer 2010)

A		Time after inoculation[a]				
Crop	Irrigation type	2	7	10	AW	AS
Mizuna	Sprikle	1.87_{A}	1.88_{A}	(1/3)	(2/3)	(2/3)
	Drip	2.45_{A}	(3/3)[b]	(1/3)	(1/3)	(2/3)
Tatsoi	Sprikle	1.80_{A}	(3/3)	(3/3)	(3/3)	(3/3)
	Drip	2.17_{A}	(3/3)	(2/3)	(1/3)	(2/3)
Red	Sprikle	2.16_{A}	(3/3)	1.57_{A}	(3/3)	(3/3)
Chard	Drip	1.71_{A}	(3/3)	(2/3)	(2/3)	(2/3)
B		Time after inoculation[a]				
Crop	Irrigation type	2	7	10	AW	AS
Mizuna	Sprikle	1.66_{A}	(3/3)[b]	1.73_{A}	1.65_{A}	1.77_{A}
	Drip	1.95_{A}	(3/3)	1.69_{A}	(3/3)	(3/3)
Tatsoi	Sprikle	2.27_{A}	2.46_{A}	2.06_{A}	1.76_{A}	(3/3)
	Drip	$*3.37_{A}$	2.82_{A}	3.39_{A}	2.72_{A}	2.30_{A}
Red	Sprikle	2.10_{A}	(3/3)	(3/3)	(3/3)	(3/3)
Chard	Drip	2.97_{A}	(3/3)	$*1.85_{AB}$	1.85_{B}	1.79_{B}

[a] Levels of *E. coli* O157:H7 are expressed as log CFU/g (n=3).

[b] Ratio denotes the number of replications tested positive for *E. coli* O157:H7 through amplification of *rfbE* by qRT-PCR per total.

Different capital letters denote significant difference ($p<0.05$) within the same row.

* Denotes significant difference ($p<0.05$) between the irrigation type for the same crop and time point

Table 5. Persistence of *E. coli* (A) and *E. coli* O157:H7 (B) inoculated on Mizuna, Tatsoi and Red Chard baby leaves from production to processing and storage (fall 2010).

A		Time after inoculation[a]				
Crop	Leaf age	2	7	10	AW	AS
Mizuna	3 weeks	2.51_{A}	1.82_{B}	1.76_{B}	(3/3)[b]	(3/3)
Tatsoi		3.25_{A}	1.52_{B}	1.74_{B}	1.50_{B}	(3/3)
Red Chard		2.16_{A}	2.22_{A}	2.34_{A}	2.08_{A}	1.86_{A}
Mizuna	4 weeks	3.47_{A}	(3/3)	1.75_{B}	(3/3)	(3/3)
Tatsoi		3.33_{A}	$*2.76_{B}$	1.75_{C}	(3/3)	(3/3)
Red Chard		$*3.11_{A}$	2.83_{AB}	2.56_{AB}	2.32_{AB}	2.07_{B}

B		Time after inoculation[a]				
Crop	Leaf age	2	7	10	AW	AS
Mizuna	3 weeks	3.15_{A}	(3/3)[b]	1.74_{B}	(3/3)	(1/3)
Tatsoi		2.69_{A}	1.75_{B}	(3/3)	1.93_{AB}	(2/3)
Red Chard		3.91_{A}	2.46_{B}	2.31_{B}	2.28_{B}	2.07_{B}
Mizuna	4 weeks	2.68_{A}	$*2.02_{B}$	1.91_{B}	1.69_{B}	(1/3)
Tatsoi		3.32_{A}	2.01_{B}	(3/3)	(2/3)	(2/3)
Red Chard		3.37_{A}	2.18_{B}	2.28_{B}	2.22_{B}	1.54_{B}

[a] Levels of *E. coli* are expressed as log CFU/g (n=3).

+, denotes positive sample

[b] Ratio denotes the number of replications tested positive by selective enrichment in TSB+rif and confirmed by surface plating on CHROMECC+rif for generic *E. coli* or, through amplification of *rfbE* by qRT-PCR for *E. coli* O157:H7 per total. Limit of detection is 1.43 log CFU/g.

Different capital letters denote significant difference ($p<0.05$) within the same row.

* Denotes significant difference ($p<0.05$) between the leaf age for the same crop and time point.

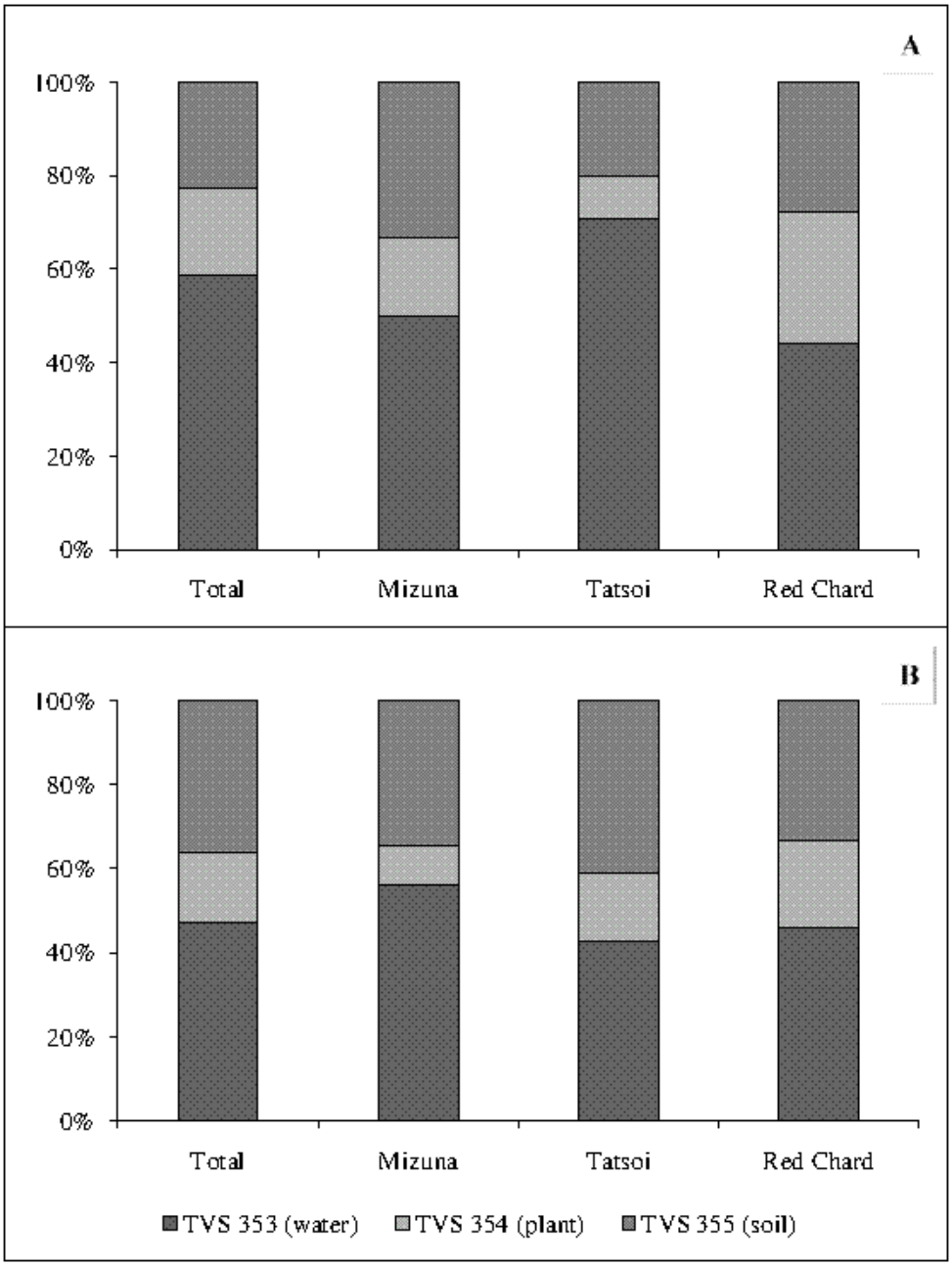

Figure 1. Relative abundance of E. coli strain TVS 353, TVS 354 and TVS 355 in the phyllosphere of Mizuna, Tatsoi and Red Chard during the summer (A) and fall (B) trial.

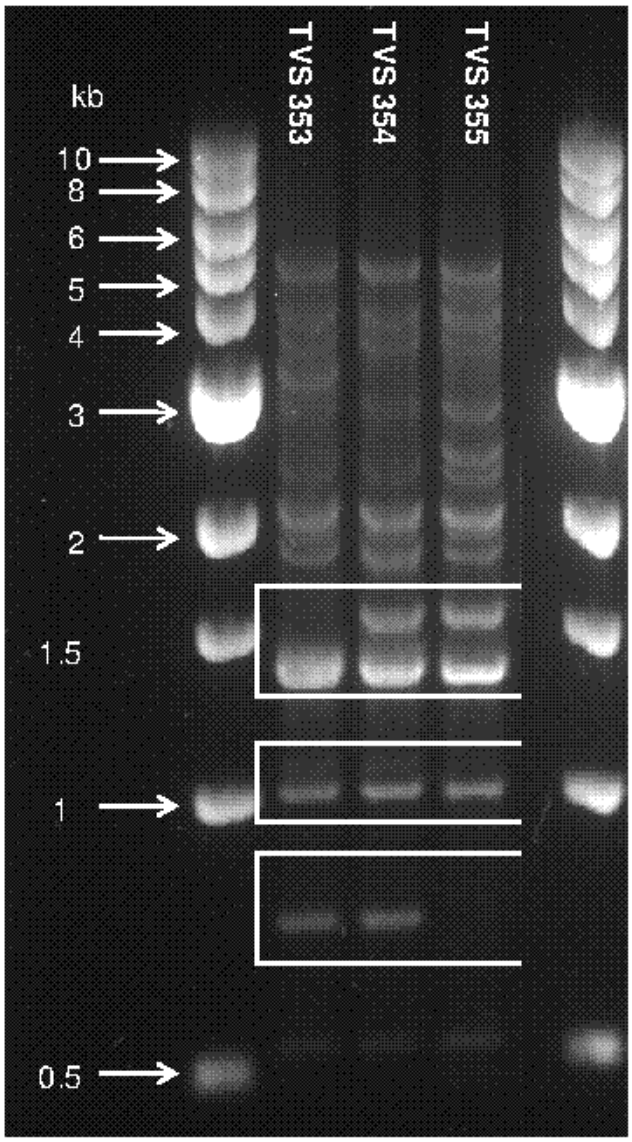

Figure 2. A PCR fingerprint patterns of *E. coli* strains TVS 353, TVS 354 and TVS 355 strains by REP-PCR.

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CHAPTER 6

Combined effect of UV-C pretreatment and high oxygen packaging on overall quality of fresh-cut Tatsoi baby leaves

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6.1. INTRODUCTION

Asian baby leaves vegetables have recently grown in popularity as a base ingredient for fresh-cut mixed salads. Among those Tatsoi (*Brassica rapa* cv. rosularis), Mizuna (*Brassica rapa* cv. japonica) and Red Chard (*Beta vulgaris* cv. cycla) are the most important. Tatsoi is a variety of *Brassica rapa* grown for greens. The leaves are spoon-shaped, dark green, and waxy, with a short green petiole (2-9 cm) on the leaf stalk. Tatsoi leaves have a soft creamy texture and has a subtle yet distinctive flavor, being a valuable source of phosphorous, calcium iron, potassium, and vitamins A and C (Martin, 2008). It is well known that the association of a diet rich in fruit and vegetables with the prevention of chronic diseases such as cancer or cardiovascular diseases which have stimulated the interest in this kind of products and their bioactive compounds (Steinmetz and Potter, 1996; Liu, 2003). Consumers are concerned about the relationship between the food they eat and their overall health and well-being. This fact has increased the demand of convenient fresh food, keeping their living fresh state, free from additives, with high nutritional value and powerful antioxidant properties which fight reactive oxygen species (ROS). The industry satisfies this particular demand by offering minimally fresh processed or fresh-cut fruit and. Fresh-cut vegetables are ready-to-eat products which fit very well within the new consumer trends. These commodities are elaborated free from additives by using light combined methods such as washing, cutting, disinfecting, and packaged at chilling temperature under polymeric films able to generate optimum modified atmosphere packaging (MAP) conditions (Artés et al., 2009).

Polyphenols contribute to flavour natural food pigments and stress resistance of vegetables (Lule and Xia, 2005). Phenolic compounds can act as antioxidants (Shahidi and Naczk, 1995) by interfering with oxidation processes through chain-breaking reaction activities (primary oxidation) or through scavenging of free radicals (secondary oxidation) (Gordon, 1990). Phenolic compounds and vitamin C are the major antioxidants sources of *Brassica* vegetables, due to their high content and high antioxidant activity. Lipid-soluble antioxidants (carotenoids and vitamin E) are responsible for up to 20% of the total antioxidant activity (Podsedek, 2007). Antioxidant systems in plants prevent or mitigate the membrane peroxidation resulting from ROS under abiotic stress conditions (Korkmaz et al., 2010). Yellowing or green colour loss is a natural phenomenon during plant senescence as a consequence of chlorophyll degradation. Several studies have reported the relationship of this pigment with the prevention of cancer (Egner et al., 2001; Egner et al., 2003; De Vogel et al., 2005) as well as their absorption by humans (Ferruzzi and Blakeslee, 2007).

Innovative MAP under superatmospheric O_2 conditions and UV-light has been proposed as innovative sustainable sanitation strategies which can be used for keeping quality and safety of several fresh-cut produces (Allende and Artés, 2003; Rico et al., 2007; Artés et al., 2009). Non-conventional active MAP is a novel technique for packaging plant commodities which replace the original atmospheric gas partial

pressure with noble gases (He, Ar or Xe), N_2O, N_2, or superatmospheric O_2 levels that might be favourable for keeping its overall quality (Tomás-Callejas et al., 2011a). The effects of superatmospheric O_2 MAP on quality changes in several leafy vegetables have been early reported (Jacxsens et al., 2001; Allende et al., 2004), although it needs to be supported by more research. The use of non-ionizing, germicidal and artificial ultraviolet light (UV) at a wavelength of 190–280 nm (UV–C) could be effective for surface decontamination of fresh-cut products (Artés-Hernández et al., 2009; Artés-Hernández et al., 2010). UV treatment can be performed at low temperature and is classified as a non-thermal disinfection method (Tran and Farid, 2004). Besides the antimicrobial effect, it is also hypothesized that selected abiotic stresses such as UV-C light would affect the secondary metabolism of fresh produce and could increase the synthesis of phytochemicals with nutraceutical activity (Cisneros-Zevallos, 2003).

Food integrated preservation techniques such as sanitizing treatments, MAP and chilling storage temperatures are commonly applied to avoid the quality deterioration of horticultural produces. This deterioration can be caused by microorganisms and/or by a variety of physico-chemical reactions that take place after harvesting. Following the principle of 'hurdle technology', a combination of various preservation techniques at lower individual intensities can have additive or even synergistic antimicrobial effects, while their impact on sensory and nutritive properties of the food is minimized (Leistner, 1992; 2000).

The main objective of the present work was to evaluate the effect of a moderate UV-C pretreatment, a superatmospheric O_2 MAP and its combination compared to a conventional passive MAP on microbial, nutritional and sensory changes of fresh-cut Tatsoi baby leaves during shelf life.

6.2. MATERIALS AND METHODS

6.2.1. Plant material

Tatsoi (*Brassica rapa* var. narinosa) baby leaves were commercially air grown under Mediterranean climate (Campo de Cartagena, Murcia, Spain) and mechanically harvested at a marketable development stage. Immediately after harvesting the leaves were transported to the Technical University of Cartagena where they were air pre-cooled at 5ºC until they were processed the next day.

6.2.2. Sample preparation, treatments and storage conditions

Baby leaves were minimally processed in a disinfected cold room at 8ºC and those with defects were discarded. Sound Tatsoi leaves were prewashed for 1 min with tap water at 5ºC and then the following treatments were applied:

- T1 (Control): leaves were washed in tap water for 2 min at 5ºC and then spin dried for 45 s in a handheld salad spinner (Dynamic Professional, Vence,

France) to eliminate water excess. Leaves were packaged under passive MAP as described below.

- T2 (NaClO): leaves were washed with a standard industrial disinfection treatment with 100 mg/L NaClO at pH 6.5 for 2 min at 5ºC, rinsed with tap water for 1 min and then spin dried to eliminate water excess. Leaves were packaged under passive MAP as described below.
- T3 (UV-C): prewashed leaves were spin dried to eliminate excess water and subsequently were subjected to 4.54 kJ UV-C m-2 radiation. Leaves were packaged under passive MAP as described below. The UV-C dose applied was selected based on previous reports and our preliminary experiments in fresh leafy vegetables. The UV-C equipment used was fully described in Artés-Hernández et al. (2009).
- T4 (O_2-enriched MAP): prewashed leaves were spin dried to eliminate water excess and subsequently were packaged under active MAP with an initial 100 kPa O_2 concentration as described below.
- T5 (UV-C + O_2 -enriched MAP): this treatment was a combination of T_3 + T_4.

For all treatments 40 g of leaves were placed in 1,500 mL polypropylene (PP) baskets which were thermally sealed at the top with a bi-oriented PP (BOPP) film of 40 µm thickness to generate a passive MAP. The active O_2-enriched MAP was generated just after flushing the O_2 within the packages and sealed at the top with the BOPP film. The O_2 and CO_2 transmission rates at 23ºC and 0% RH of BOPP were similar with 800 cm^3 /m^2d atm and the water vapour transmission rate at 23ºC and 85% RH was 0.7 g /m^2d atm (data provided by the supplier, Plásticos del Segura S.L., Murcia, Spain). Five replicates of one basket per treatment and storage duration (processing day and after 4, 6, 8 and 11 days) were prepared and stored in a dark cold room at 5ºC.

6.2.3. Respiration rate and gas composition within packages

The respiration rate (RR) of control leaves was determined at 5ºC by using a closed system. Three replicates of 20 g leaves were placed within 750 mL glass jars at 5ºC up to 7 days. The increases in CO_2 were monitored almost daily starting at 2.5 hours after having closed the jars. Headspace gas samples (1 mL) were withdrawn from the jars with a gas-tight syringe and analyzed in a gas chromatograph (Thermo Finnigan Trace GC, Milan, Italy), equipped with a thermal conductivity detector (150 ºC), oven (ramp from 40 to 90 ºC), injector (150 ºC) and Poropack-N 80/100 column. He (20 mL /min) was the carrier gas. In order to avoid CO_2 accumulation and to maintain a high RH within jars, a continuous flow of 30 mL/min of humidified air was applied between each observation. Gas partial pressures (O_2 and CO_2) within packages were monitored on the processing day and after 4, 6, 8 and 11 days of storage at 5ºC by using a gas-analyzer (PBI Dansensor, CheckPoint, Ringsted, Denmark). In order to avoid modifications in the headspace due to gas sampling, each analyzed package for each evaluation time was opened and used for further analysis. Three replicates of one basket were used for each treatment and evaluation period.

6.2.4. Colour

Leaf colour was monitored at three equidistant points on the upper side of leaves using a compact tristimulus colorimeter (Minolta CR-300, Ramsey, NJ, USA) with an 8 mm diameter viewing aperture and a white plate C reference (Y = 94.3, x = 0.3142, y = 0.3211, standard CIE illuminant, 2° observer). Values were expressed as Hunter lightness, chroma and hue angle parameters. Observations were made on thirty randomly selected leaves. Measurements in all treatments were replicated three times.

6.2.5. Sensory evaluation

A seven person panel (aged 24-62) trained in sensory quality analyses performed the evaluation. Before running the experiments a consensus was reached between the panellists in order to select those attributes that better described sensory changes. Sensory evaluation was evaluated on the processing day and after 4, 6, 8 and 11 days at 5°C. Dehydration, browning, off-odours and off-flavours were scored on a five-point scale of damage incidence and severity (1 = none, 2 = slight, 3 = moderate, 4 = severe and 5 = extreme). Visual appearance and overall quality was evaluated by using a nine-point scale (1 = extremely poor, 5= fair, limit of usability, 9 = excellent).

6.2.6. Microbiological analysis

For determining the microbial growth standard enumeration methods were used. Three random samples were taken on each evaluation time. Ten grams of leaves were homogenized in 90 mL of sterile buffered peptone water (BPW) (Scharlau Chemie SA, Barcelona, Spain) for 1 min in a sterile stomacher bag (Model 400 Bags 6141, London, UK) using a Masticator (Colwort Stomacher 400 Lab, Seward Medical, London, UK). In order to determine the enumeration of each microbial group (mesophilic, enterobacteria, psychrotrophic, yeasts and moulds), ten-fold dilution series were prepared in 9 mL of BPW. The following media and incubation conditions were used: plate count modified agar (Scharlau Chemie, Barcelona, Spain) for mesophilic and psychrotrophic aerobic bacteria, incubated at 30 °C for 48 h and at 5 °C for 7 days respectively; violet red bile dextrose agar (Scharlau Chemie, Barcelona, Spain) for enterobacteria, incubated at 37 °C for 48 h; and potato dextrose agar base (Scharlau Chemie, Barcelona, Spain) with 100 mg/L oxytetracycline (Sigma Chemical Co., St Louis, MO, USA) for yeasts and moulds, incubated for 3-5 days at 22 °C. All microbial counts were reported as log colony forming units per gram (log CFU/g). The presence of *Salmonella* spp., *Listeria monocytogenes* and generic *Escherichia coli* was also evaluated according to the EU legislation for fresh-cut vegetables (Regulation EC 1441/2007, 2007). All analyses were made in triplicates.

6.2.7. Chlorophyll content

For tissue preparation, about 25 g of leaves from each treatment were frozen in liquid N_2, ground using a mincer (IKA, A 11 basic, Berlin, Germany) at 28,000 x g for

10 s, and stored at-80°C. The sample preparation for chlorophyll determination was conducted according to Smith and Benítez (1955). The total chlorophyll pigments were extracted with hexane. A 0.5 g sample of frozen shredded leaves was mixed with 9 mL of hexane and 15 mL of a mixture of methanol:acetone (1:2). The extraction was carried out for 5 h at 5°C in darkness. Then the samples were shaken at 200 x g every 15 min by using a vortex (Velp Scientifica, Zx^3, Milano, Italy). After incubation, 25 mL of 1M NaCl was added. Subsequently the samples were shaken again and then centrifuged for 30 min at 2,800 x g at 4°C. After centrifugation, 1 mL of the supernatant was pipetted into a quartz cuvette (Hellma GmbH & Co., Müllheim, Germany). The equations developed by Wellburn (1994) were used to determine the individual levels of both chlorophyll a (Chl *a*) = 10.05 A_{662} – 0.766 A_{644} and chlorophyll b (Chl *b*) = 16.37 A_{644} – 3.14 A_{662} where total chlorophylls amount was calculated as Chl *a* + Chl *b*. The absorbance (*A*) at 662 and 644 nm was measured using a UV-visible spectrophotometer (Hewlet Packard, model 8453, Columbia, USA). Chlorophyll content was expressed as mg Chl/kg fresh weight (fw). All measurements were made in triplicates.

6.2.8. Total phenolics content

Frozen samples of 0.5 g were homogenized with 3 mL of methanol/water (4:1 v/v) in an Ultraturrax (Janke & Kunkel, Ika - Labortechnik, Germany) at 24,000 x g for 1 min. Next, the tubes were placed on an ice bed, in darkness and were homogenized with a vortex at time zero, after 30 min and after 1 h. Afterwards 2 mL sample was transferred in 2 x 2 mL eppendorfs and centrifuged at 15,000 x g for 10 min at 4°C. The supernatant was used as an extract for each sample. The amount of total phenolic compounds was determined as described previously by Singleton and Rossi (1965). A 100 µL aliquot of extract was mixed with 150 µL of Foli-Ciocalteu reagent (1:1 v/v, diluted with milli-Q-water) and incubated for 1 min before 1 mL of 75 g/L sodium carbonate (2 % w/v) + NaOH (0.4 % w/v) was added. The mixture was then incubated for 1.5 h at room temperature in darkness measuring the absorbance at 765 nm (Hewlet Packard 8453, UV-Vis spectrophotometer, Columbia, USA). Total phenolics content was expressed as chlorogenic acid equivalents (ChAE) in mg/kg fw. All extracts were analyzed in triplicates.

6.2.9. Total antioxidant activity

The antioxidant activity of the fresh-cut Tatsoi leaves was based on the evaluation of the free radical scavenging capacity according to Brand-Williams et al., (1995). A solution of 0.7 mM 2,2-diphenil-1-picrylhydrazil radical in methanol was prepared. An aliquot of 25 µL of the extract obtained from the preparation of phenolic compounds was added to 975 µL of this solution. The mixture was incubated in darkness for 1 h at room temperature. The antioxidant activity was measured by decreasing the absorbance at 517 nm (Hewlet Packard 8453, UV-vis spectrophotometer, Columbia, USA). Results were expressed as mg ascorbic acid equivalent antioxidant capacity (AEAC) /kg fw. All measurements were made in triplicates.

6.2.10. Statistical analysis

The experiment was based on a 5 x 4 bifactorial (treatment x storage time). For the statistical analysis the Statistical Analyses System (SAS) 9.2. (SAS Institute, Cary, NC, USA) was used. Replication of each treatment and storage time combination was repeated in triplicate. Comparison among treatments was carried using the MIXED procedureand Tukey's multiple comparisons for mean separation. Statistical significance was established when $p < 0.05$. All data was previously evaluated for normality and homogeneity of variance using the UNIVARIATE procedure of SAS.

6.3. RESULTS

6.3.1. Respiration rate and gas composition within packages

The RR of untreated Tatsoi leaves was monitored up to 8 days at 5ºC. In spite of a slight initial increase in RR, due to the stress induced by the processing, it was remained quite stable after 3 days at 5ºC at about 20-25 mg CO_2 /kg h (data not shown).

The gas partial pressure changes of O_2 and CO_2 within packages throughout 11 days at 5ºC are shown in Fig. 1. The initial gas composition of passive MAP was air (20,9 kPa O_2 and 0.03 kPa CO_2). As expected, due to the respiratory activity of the leaves and the film permeability, the O_2 levels decreased and the CO_2 increased during shelf life. No significant differences ($p<0.05$) were found among the control and NaClO treatments. However, differences among UV-C in comparison to NaClO and control treatments after 4 days at 5ºC were found. Equilibrium gas partial pressures within packages for passive MAP were reached after 8 days at 5ºC with 5-6 kPa O_2 + 15-16 kPa CO_2 (balanced with N_2) for control and NaClO treatments and 1-2 kPa O_2 + 19-20 kPa CO_2 for UV-C pre-treated samples (Fig. 1A). For the active MAPs, gas partial pressures after 8 days at 5ºC reached around 38-40 kPa O_2 + 18-20 kPa CO_2 (Fig. 1A). As observed before for the passive MAPs, higher CO_2 and lower O_2 values were registered again for the samples exposed to UV-C radiation.

6.3.2. Colour

As a general trend for all treatments maintenance of lightness (L*) during the shelf life was monitored and no significant differences among treatments were found (Table 1). However, the combination of UV-C + O_2-enriched MAP resulted in a significant decrease of L* after 11 days at 5ºC with respect to the initial values, although L* differences lower than 3 units are not detectable by the human eye. Regarding to the Chroma parameter (C*), the initial value was 20.3 and was kept quite constant regardless of the treatment throughout 11 days at 5ºC. Similar behaviour was observed for Hue angle where values around 179 remained constant during the shelf life for all treatments (data not shown).

6.3.3. Sensory evaluation

After 6 days at 5ºC, Tatsoi baby leaves did not show noticeable symptoms of dehydration, browning, off-flavours or off-odours that exceeded the acceptable limit of usability for fresh consumption (Fig. 2). However, all samples showed an important decrease of their overall sensory quality after 11 days at 5ºC being not acceptable for fresh consumption and visual appearance showed the same pattern (data not shown). According to overall sensory quality, a maximum shelf life of 9 to 10 days at 5ºC was established in our conditions. In particular, after the determined shelf life, moderate dehydration symptoms occurred and slight to moderate off-odours were observed (Fig. 2). Slight browning and off-flavours were registered after shelf life (data not shown).

6.3.4. Microbiological analysis

The initial microbial load for total aerobic mesophilic (TAM) bacteria was 3.7 log CFU/g (Fig. 3A). Washing with chlorinated water resulted in 0.8 log reductions and in the leaves subjected to UV-C light the initial TAM load decreased in 0.6 log CFU/g when compared to the control. NaClO treatment kept the TAM reductions in 0.7, 0.8 and 0.4 log CFU/g after 4, 6 and 8 days at 5ºC compared to the control. After 11 days at 5ºC TAM load was similar for the control and NaClO treated samples. During the first 4 days at 5ºC slight increases in TAM counts for the O_2-enriched MAP treatment were found. Then, the antimicrobial effect of the superatmospheric O_2 kept the TAM population quite stable during the shelf life. Significant differences on TAM counts among UV-C treatment and the control and NaClO were found. The decontamination effect of UV-C reduced the initial TAM population in 0.6 log CFU/g compared to control. The antimicrobial effect of the UV-C light was kept during the shelf life and 1.1, 1.3 and 1.2 log reductions after 6, 8 and 11 days at 5ºC likened to the control were registered. The combination of UV-C + O_2-enriched MAP was even more effective than the remaining treatments and in comparison to control reductions of 1.7, 1.8 and 1.9 log after 6, 8 and 11 days respectively were found.

Regarding *Enterobacteriaceae* (Fig. 3B), no differences among treatments in counts on the processing day were observed. The enterobacteria population increased from 2.2 to 4.0 log CFU/g in control leaves during the shelf life and slight increases were registered for NaClO and O_2-enriched MAP treatments. The samples pre-treated with UV-C light and its combination with O_2-enriched MAP kept the enterobacteria load around the limit of detection (2 log CFU/g) throughout the shelf-life.

All psychrophilic counts were below the limit of detection (2 log CFU/g) on the processing day (Figure 3C). The UV-C pre-treatment and the UV-C+O_2-enriched MAP treatments inhibited psychrophilic growth and a bacteriostatic effect was observed during the first 8 days at 5ºC. However, after 11 days no significant differences among treatments were found.

Washing with chlorinated water reduced the initial population of yeast and moulds in approximately 1.8 log CFU/g compared to the control (Fig. 3D). An inhibitory effect in UV-C + O_2-enriched MAP treatment was found and yeast and moulds counts in the control, O_2-enriched MAP and UV-C treated samples remained quite constant up to 11 days at 5ºC.

Generic *E. coli*, *Salmonella* spp., and *Listeria monocytogenes* were not recovered from any of the samples, being then all safe according to the EC legislation (Regulation EC 1441/2007, 2007).

6.3.5. Chlorophyll content

The initial chlorophyll amount was in the order of 720 mg/kg fw (Table 2). The Chl *a* content represented around 70% and Chl *b* around 30% of the total chlorophyll content. The general trend was to decrease the initial chlorophyll content throughout the shelf-life for all treatments. UV-C pre-treated samples showed lower total chlorophyll content after shelf life in comparison to the remaining treatments. However, all these variations among storage time and treatments were no statistically significant.

6.3.6. Total phenolic content

Total polyphenols content of fresh-cut Tatsoi baby leaves at harvest was 225 mg ChAE/kg fw. After 4 days at 5ºC the polyphenols content significantly increased and was kept throughout the shelf life for all treatments (Table 2). After 11 days the total phenolic content ranged between 285 – 316 mg ChAE/kg fw, with an increase of 12-29% of the initial value, and no differences among treatments were found.

6.3.7. Total antioxidant activity

The total antioxidant activity of fresh-cut Tatsoi baby leaves at harvest was 638 mg AEAC/kg fw (Table 2). As observed for total phenolic content, this value strongly increased (25-40%) after 4 days at 5ºC for all treatments without differences among them. Then, total antioxidant activity progressively decreased reaching values after 11 days similar to the initial content. The control treatment showed the lowest antioxidant activity (564 mg AEAC/kg fw) with respect to the remaining treatments which ranged between 661-698 mg AEAC/kg fw after 11 days at 5ºC without differences among them.

6.4. DISCUSSION

The microbial safety and stability as well as the sensory and nutritional quality of food are based on an application of combined preservative agents (Leistner, 2000). For fresh-cut commodities it implies several factors from a well designed integrated production, handling and processing to proper distribution chains, keeping appropriate chilling storage temperatures and optimal MAP conditions throughout the entire

commercial life (Artés et al., 2009). Research on the combination of different food preservation techniques has increased because many of them act by amplifying this interference with homeostasis (Gould, 1996). Innovative superatmospheric O_2 MAP and UV-C pretreatment techniques have been proposed as sustainable sanitation strategies which can be used for keeping quality and safety of fresh-cut produces (Allende and Artés, 2003; Soliva-Fortuny and Martin-Belloso, 2003; Allende et al., 2004; Rico et al., 2007).

Baby leaves vegetables are very susceptible to mechanical damage, weight loss, and microbial growth which strongly affect the shelf life. Fresh-cut produces usually contain microorganisms such as psychrotrophic aerobic bacteria, coliforms, yeast, moulds and pectinolytic bacteria. Commonly total counts of microbiological populations on minimally processed vegetables after processing range from 3 to 6 log CFU/g (Ragaert et al., 2007). In the current study, reductions of 0.8 and 0.6 log CFU/g of TAM bacteria for the NaClO and UV-C treatments compared to the control were achieved. This result agrees with our previous results in where reductions of TAM from 0.3 to 1 log CFU/g in baby spinach leaves subjected to 0 to 7.94 kJ m^{-2} UV-C were found (Artés-Hernández et al., 2009). Other studies have reported that UV-C inhibited microbial growth and delay senescence of zucchini squash (Erkan et al., 2001), carrots (Mercier and Arul, 1993), sweet potato (Stevens et al., 1999) and fresh-cut melon (Manzoco et al., 2010). The O_2-enriched MAP showed a beneficial effect for inhibiting the TAM growth during the shelf life. This inhibitory effect could be related with the toxicity of high O_2 concentrations to cells (Wszelaki and Mitcham, 2000), which may induce DNA and nucleoprotein damage, as well as general protein damage in microorganism (Moradas-Ferreiras et al., 1996). The combined effect of high O_2 concentrations (over 60 kPa) and CO_2 around 10 to 20 kPa may provide adequate suppression of microbial growth increasing the shelf-life (Jacxsens et al., 2001; Allende et al., 2004; Geysen et al., 2006).

Many research studies reported the antimicrobial effect of the disinfectant agents only after their application. However, the maintenance of the microbiological reduction reached during storage is even more important. The UV-C light dose was selected based on our previous findings. An important point to take into account is that 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). Artés-Hernández et al. (2009) reported that UV-C doses above 5 kJ m^{-2} did not show a clear inhibitory effect on microbial growth on minimally processed spinach during the chilling storage at 5 and 8ºC. Similar results were reported by Escalona et al. (2010) where short exposures times and low UV-C radiations doses were effective to reduce epiphytic microflora associated to baby spinach leaves up to 14 days at 5ºC. An inadequate UV-C light management on leafy greens might damage the superficial tissues of the leaves and could release nutrients and promote microbiological growth throughout shelf life. Charles et al. (2008) demonstrated evidence that UV-C light affects the morphology of tomato fruit surface. It has been suggested that UV-C light

could alter the cell permeability and may have resulted in a greater leakage of electrolytes, aminoacids, and carbohydrates which might stimulate microbial growth (Nigro et al., 1998). In addition, it might impact on the sensory quality. The crucial point is whether a safe dose can be found which would greatly impair microbial growth without damaging the product (Ben-Yehoshua and Mercier, 2005).

While the enterobacteria population remained quite constant throughout shelf life for all treatments except in control leaves; UV-C and the combined UV-C+ O_2-enriched treatment were those which best controlled the psychrotrophic growth. Our results agree with those reported by Allende and Artés (2003) where the combination of passive MAP and low UV-C radiations reduced psychrotophic populations about 0.5 – 2 log CFU/g on the processing day and during the shelf life. O_2-enriched MAP showed an inhibitory effect of psychrotophic growth in fresh-cut Red Chard stored up to 8 days at 5ºC (Tomás-Callejas et al., 2011a). However, this behaviour was not observed for fresh-cut Tatsoi. The effect of high O_2 concentrations (95 kPa O_2) on the psychrotrophic human pathogen *Listeria monocytogenes* growth, which is associated with refrigerated fresh-cut vegetables, resulted in an extended lag phase (Jacxsens et al., 2001).

Regarding the nutritional profile, the different polyphenol contents in *Brassica* vegetables may be influenced by several factors, including genetic and environmental influences, growing period and maturity stage at harvest (Naczk and Shahidi, 2006; Singh et al., 2007). Antioxidants are not only phenolic based and other compounds such as phytic acid, selenium, tocopherol, etc can contribute to the antioxidant power of plant tissues (Alothman et al., 2009). In both cases, an increase of total phenolic content and total antioxidant activity was registered after 4 days at 5ºC. The wound-induced phenomenon in the phenolic metabolism could explain this behavior. However, enzymatic activities such as PAL activity should be tested to corroborate the hypothesis. Then, the values remained quite constant during the shelf life. These results agree with our previous findings on fresh-cut Mizuna baby leaves, a different cultivar of *Brassica rapa*, where phenolic content was kept throughout the shelf life after minimal processing (Tomás-Callejas et al., 2011b). No significant changes on total chlorophyll content were registered during the shelf life which is directly related to the results of colour parameters where no significant changes in L*, C* and Hue angle were found. Colour changes of fresh-cut leafy vegetables during storage can be affected by storage under light or dark conditions (Ferrante et al., 2004).

Gas partial pressures within packages after 8 days at 5ºC were around 5 kPa O_2 and 15 kPa CO_2 for NaClO and control treatment. O_2 levels close to the theoretical limit of anaerobic respiration were registered for the passive MAP subjected to UV-C light. This behaviour is due to the induced stress caused by UV-C light which accelerates respiratory activity, as previously reported for fresh-cut lettuce and baby spinach (Allende and Artés, 2003; Escalona et al., 2010). The same trend was observed with the O_2-enriched MAP and the combination of O_2-enriched MAP+UV-C, where the irradiated samples produced higher CO_2 levels during the shelf-life.

In summary, UV-C pretreatment alone or combined with superatmospheric oxygen packaging showed a better effect for controlling TAM and psychrophilic growth than the remaining treatments tested. All assayed treatments did not affect the nutritional profile and its main quality attributes were kept during the chilling storage. The shelf life at 5ºC was established in 9-10 days for all treatments. These innovative sustainable treatments can be useful tools for the industry for keeping quality and safety of the fresh-cut Tatsoi baby leaves and very probably for others leafy vegetables while minimizing water consumption and wastewater discharge rates. Scientific data about the effects of innovative MAP on overall quality of fresh-cut baby leaves is scarce and relatively unclear. In this way, the current experiments improve the lack of information regarding the effect of high O_2 active MAP combined with UV-C pre-treatment on overall quality parameters of fresh-cut Tatsoi baby leaves.

In agreement with Leistner (2002) and the principles of the hurdle technology, an adequate management of the combination of preservation techniques can have additive or even synergistic antimicrobial effects minimizing their impact on sensory and nutritive properties. Therefore, further studies in different conditions should be performed to support this claim.

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FIGURES AND TABLES

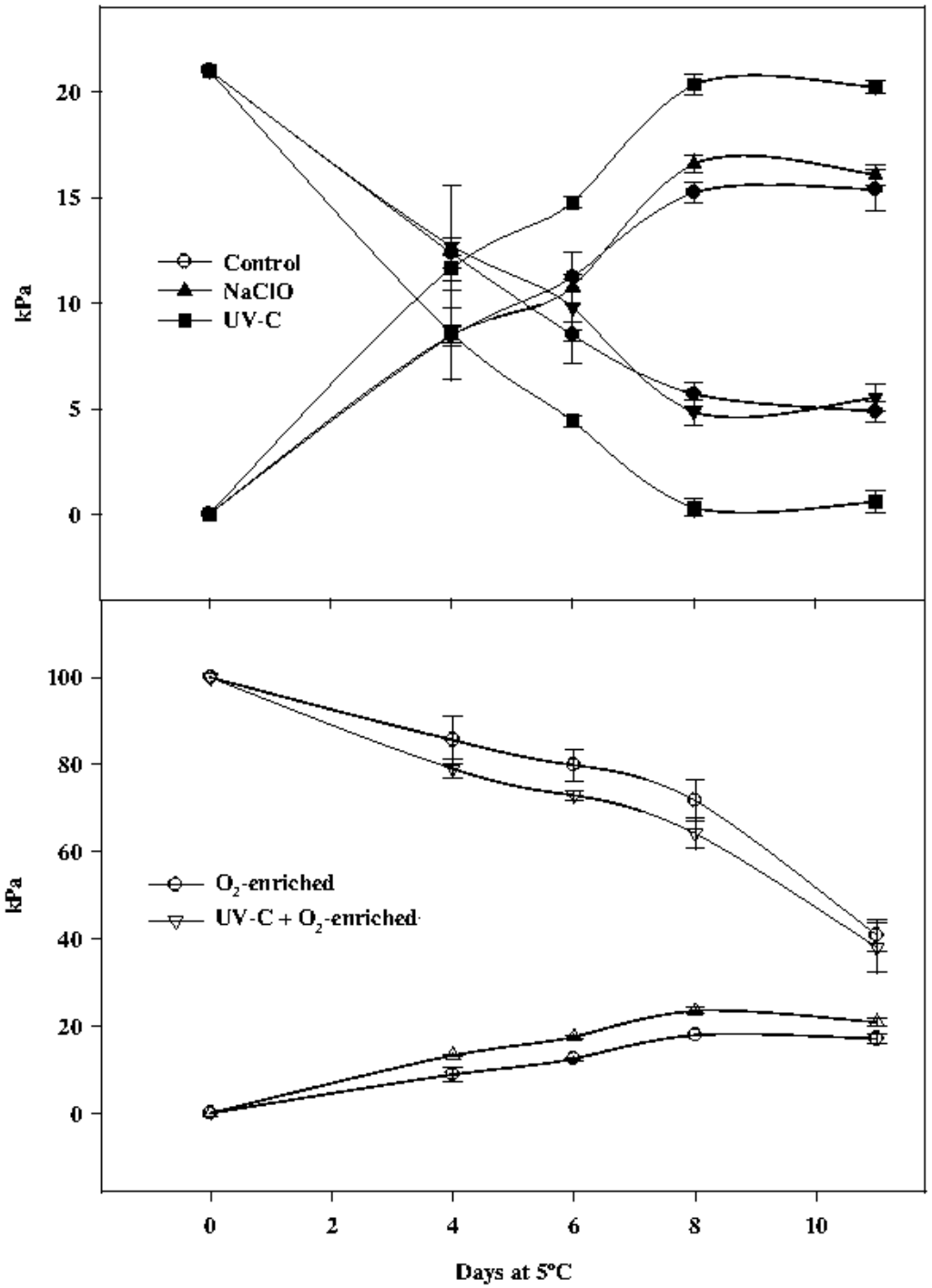

Figure 1. Gas changes within packages of fresh-cut Tatsoi baby leaves subjected to several treatments and MAP stored up to 11 days at 5°C. Error bar shows standard deviation (SD).

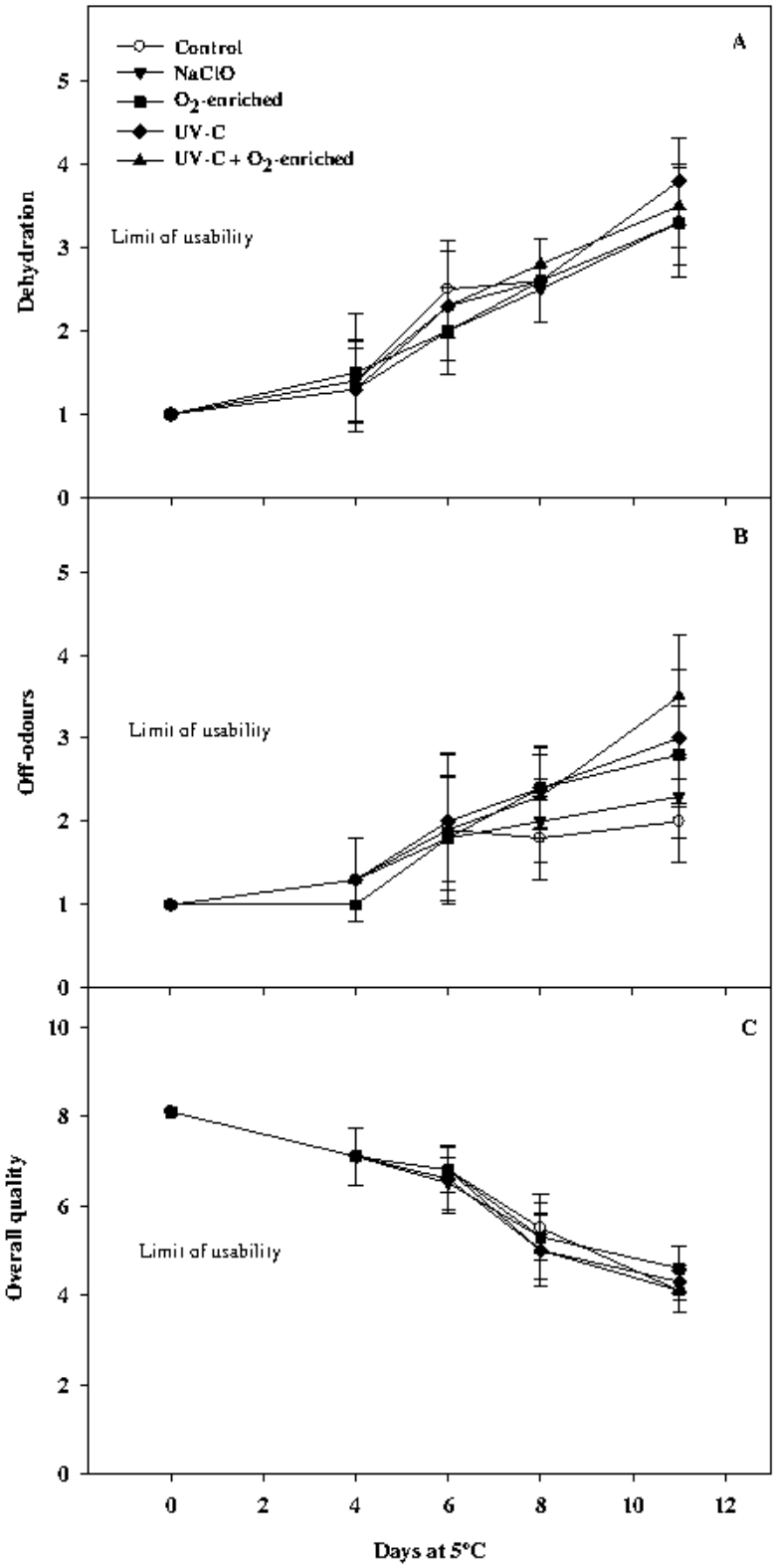

Figure 2. Sensory evaluation of dehydration (A), off-odours (B) and overall quality (C) of fresh-cut Tatsoi baby leaves subjected to several treatments and MAP stored up to 11 days at 5ºC. Error bar shows standard deviation (SD).

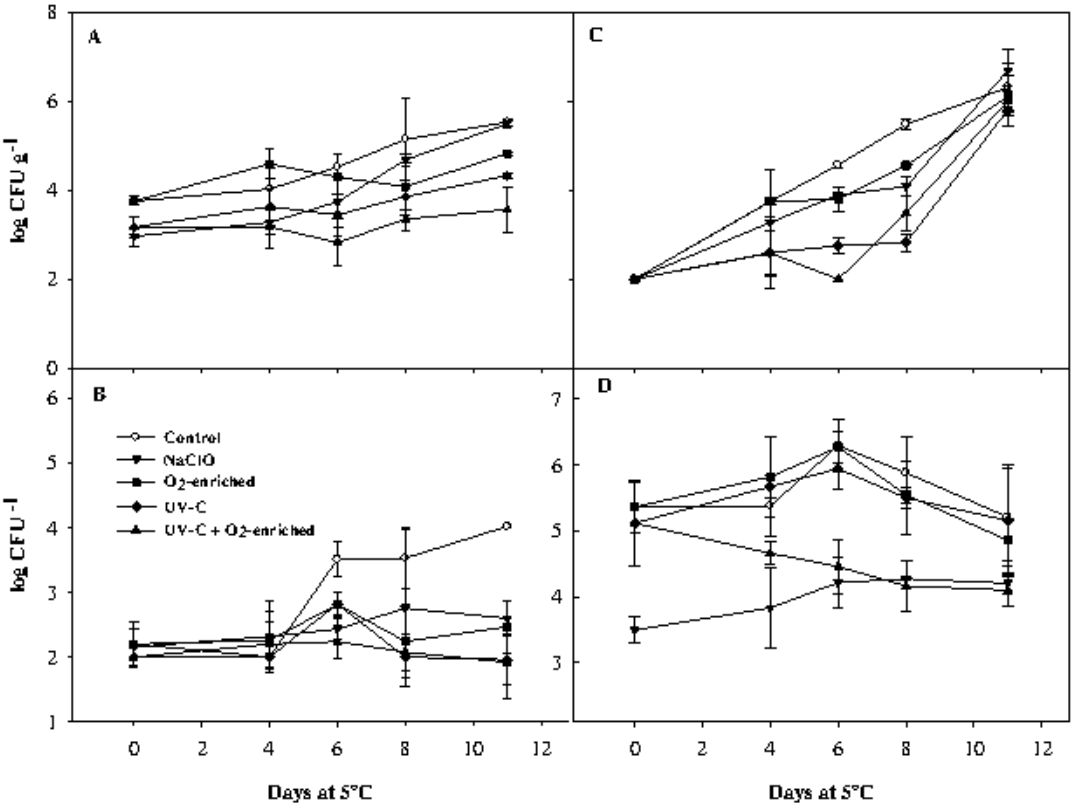

Figure 3. Mesophilic (A), enterobacteria (B), psychrophilic (C) and yeast and moulds (D) counts (log CFU/g) of fresh-cut Tatsoi baby leaves MAP stored up to 11 days at 5°C. Error bar shows standard deviation (SD).

Table 1. Colour parameters L* and C* changes for fresh-cut Tatsoi subjected to several treatments and MAP stored up to 11 days at 5ºC.

Colour parameters and treatments	Storage time at 5ºC				
	Initial	**Day 4**	**Day 6**	**Day 8**	**Day 11**
L*					
Control	38.2^{A}_{1}	39.0^{A}_{1}	36.5^{A}_{1}	37.3^{A}_{1}	38.1^{A}_{1}
NaClO	38.2^{A}_{1}	38.9^{A}_{1}	37.0^{A}_{1}	37.5^{A}_{1}	37.5^{A}_{1}
O_2-enriched MAP	38.2^{A}_{1}	38.8^{A}_{1}	37.5^{A}_{1}	37.1^{A}_{1}	36.7^{A}_{1}
UV-C	38.2^{A}_{1}	39.0^{A}_{1}	37.7^{A}_{1}	37.3^{A}_{1}	37.7^{A}_{1}
UV-C + O_2-enriched MAP	$38.2^{A}_{1,2}$	39.6^{A}_{1}	$36.9^{A}_{1,2}$	$36.8^{A}_{1,2}$	36.1^{A}_{2}
C*					
Control	20.3^{A}_{1}	18.7^{A}_{2}	21.6^{A}_{1}	21.1^{A}_{1}	22.0^{A}_{1}
NaClO	$20.3^{A}_{1,2}$	17.9^{A}_{2}	21.5^{A}_{1}	21.8^{A}_{1}	23.6^{A}_{1}
O_2-enriched MAP	$20.3^{A}_{1,2}$	18.1^{A}_{2}	21.4^{A}_{1}	21.5^{A}_{1}	20.9^{A}_{1}
UV-C	$20.3^{A}_{1,2}$	17.7^{A}_{2}	22.0^{A}_{1}	21.0^{A}_{1}	22.4^{A}_{1}
UV-C + O_2-enriched MAP	$20.3^{A}_{1,2}$	17.4^{A}_{2}	$20.1^{A}_{1,2}$	$20.3^{A}_{1,2}$	21.7^{A}_{1}

Different numbers among each row denotes significant difference ($p < 0.05$).
Different letter within each column denotes significant difference ($p < 0.05$).
Values are mean of 30 measures.

Table 2. Total chlorophyll content, total polyphenols and total antioxidant activity changes in fresh-cut Tatsoi baby leaves MAP stored up to 11 days at 5ºC.

Nutritional quality parameters and treatments	**Storage time at 5°C**				
	Initial	**Day 4**	**Day 6**	**Day 8**	**Day 11**
Total chlorophyll content (mg Chl/kg fw)					
Control	720^{A}_{1}	561^{A}_{1}	572^{A}_{1}	583^{A}_{1}	657^{A}_{1}
NaClO	720^{A}_{1}	589^{A}_{1}	564^{A}_{1}	539^{A}_{1}	665^{A}_{1}
O_2-enriched MAP	720^{A}_{1}	704^{A}_{1}	649^{A}_{1}	595^{A}_{1}	608^{A}_{1}
UV-C	720^{A}_{1}	697^{A}_{1}	654^{A}_{1}	612^{A}_{1}	562^{A}_{1}
UV-C + O_2-enriched MAP	720^{A}_{1}	642^{A}_{1}	617^{A}_{1}	526^{A}_{1}	583^{A}_{1}
Total polyphenols content (mg ChAE/ kg fw)					
Control	225^{A}_{2}	$283^{A}_{1,2}$	$250^{A,B}_{1,2}$	326^{A}_{1}	$316^{A}_{1,2}$
NaClO	225^{A}_{2}	355^{A}_{1}	$284^{A}_{1,2}$	369^{A}_{1}	346^{A}_{1}
O_2-enriched MAP	225^{A}_{2}	$312^{A}_{1,2}$	$224^{A,B}_{2}$	351^{A}_{1}	$308^{A}_{1,2}$
UV-C	$225^{A}_{2,3}$	350^{A}_{1}	$219^{A,B}_{3}$	$339^{A}_{1,2}$	$301^{A}_{1,2}$
UV-C + O_2-enriched MAP	$225^{A}_{2,3}$	331^{A}_{1}	217^{B}_{3}	$295^{A}_{1,2}$	$285^{A}_{1,2}$
Total antioxidant activity (mg AEAC/kg fw)					
Control	$637^{A}_{1,2}$	800^{A}_{1}	$633^{A}_{1,2}$	$626^{A}_{1,2}$	564^{A}_{2}
NaClO	637^{A}_{2}	884^{A}_{1}	566^{B}_{2}	667^{A}_{2}	666^{A}_{2}
O_2-enriched MAP	637^{A}_{2}	898^{A}_{1}	582^{A}_{2}	676^{A}_{2}	688^{A}_{2}
UV-C	637^{A}_{2}	868^{A}_{1}	$731^{A}_{1,2}$	$692^{A}_{1,2}$	665^{A}_{2}
UV-C + O_2-enriched MAP	637^{A}_{2}	846^{A}_{1}	$756^{A}_{1,2}$	650^{A}_{2}	698^{A}_{2}

Different numbers among each row denotes significant difference ($p < 0.05$).
Different letter within each column denotes significant difference ($p < 0.05$).

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CHAPTER 7

Establishment of critical operating standards for chlorine dioxide in disinfection of dump tank and flume water for fresh tomatoes

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7.1. INTRODUCTION

In 2010, an international group, led by the United Fresh Produce Association, with representation from all sectors of the fresh tomato supply chain released the *Food Safety Programs and Auditing Protocol for the Fresh Tomato Supply Chain* (Tomato Audit Protocol) (United Fresh, 2010). This more prescriptive commodity-specific standard and audit protocol was preceded by the U.S. Food and Drug Administration "Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables" (FDA, 2008) and the "Commodity Specific Food Safety Guidelines for the Fresh Tomato Supply Chain" (United Fresh, 2008). These programs provide recommendations for food safety practices that are intended to minimize the microbiological hazards associated with fresh and fresh-cut tomato products.

Tomatoes are frequently handled in packinghouses typically employing systems of re-circulated water, such as dump tanks and flume systems for unloading tomatoes from harvest bins or larger gondolas. The water used in these systems can become a point of cross contamination for spoilage organisms and plant pathogens which may lead to quality loss and decay, as well as human pathogens that may result in foodborne outbreaks (Harris et al., 2003). Disinfection of process water aims to inactivate or destroy pathogenic and other microorganisms. In addition, disinfection aims to prevent the transfer of these organisms from process water to produce and from one produce item to another during postharvest handling, increasing the likelihood that the produce is microbiologically safe for human consumption (Suslow, 1997). Chlorination (150 mg /L, pH 6.5-7.5, 2 min) is a current approved method for sanitization of dump tank and flume water in packinghouses and currently under state regulation for tomatoes in Florida (FDACS, 2006).

Sodium hypochlorite (NaClO) is a potent disinfectant with strong oxidizing properties. However, disadvantages related to its use include the formation of potentially hazardous disinfection-by-products, its strong pH dependence, and the potential for gas emission that may affect worker health (Ölmez and Kretzschmar, 2009). NaClO may partially oxidize food constituents to form secondary products such as chloroform ($CHCl_3$), haloacetic acids or other trihalomethanes that have known or suspected carcinogenic or mutagenic potential effect, with proven toxicity to liver and kidney tissues (Hrudey, 2009; Nieuwenhuijsen et al., 2000). Due to this, chlorine dioxide (ClO_2) has been proposed as an emerging sanitizer alternative to chlorine for the fresh and fresh-cut produce industry (Artés et al., 2009; Gómez-López et al., 2009). ClO_2 used as a disinfectant has several advantages over chlorine, including higher oxidant capacity (Benarde et al., 1967), lower reactivity with organic matter (Gordon and Rosenblatt, 2005) and high effectiveness at low concentrations (Huang et al., 1997). In addition, ClO_2 and its main by-product, chlorite (ClO_2^-), are classified as non-carcinogenic products (ATSRD, 2004; EPA, 2000; IIARC, 1991).

The effect of ClO_2 to inhibit several foodborne pathogens in different commodities has been previously reported. Concentrations of 4-5 mg/L of ClO_2 were effective to reduce *Salmonella* spp., *Escherichia coli* O157:H7 and *Listeria monocytogenes* inoculated onto cabbage, carrot, lettuce, strawberry and melon (Mahmoud et al., 2007; Mahmoud and Linton 2008; Sy et al., 2005).

In contrast with NaClO, ClO_2 was found to be effective against *E. coli* over a broader pH range (3 - 8) (Huang et al., 1997). While there are several studies related to the antimicrobial effect of ClO_2 on foodborne pathogens of concern within laboratory scale studies, insufficient data are available to assess performance at an industrial scale. In addition, few studies have been performed to determine the effect of pH, temperature and suspended matter on the disinfection efficacy of ClO_2 at both commercial and laboratory scale for fresh produce. In addition to being scarce, the available information is not clear, nor consistent among research reports.

Besides the use of a chemical sanitizer during the washing-disinfection step, the *Tomato Audit Protocol* is very specific that wash water temperature is a critical point during tomato postharvest handling. Tomatoes are a warm-season crops and high pulp temperatures can be reached during harvesting and transport to the packinghouse. Internalization of soft-rot bacteria into the stem scar is seen when tomatoes are submerged in water that is cooler than the pulp temperature of the tomatoes (Bartz, 1981; Bartz, 1982). As the tomato fruit cools, a vacuum is created causing water, and potentially pathogens suspended in the water, to be drawn into micro-wounds, pores, or other natural openings of the tomatoes. The standards and audit criteria for fresh tomatoes are that, in general, cold water immersion as a cooling technique shall not be used and, if using a dump tank, maintain water temperature 5.5°C above the incoming fruit pulp temperature to minimize the risk of intrusion of microorganisms into the tomatoes to the degree possible (Bartz, 1988; Ibarra-Sánchez et al., 2004; Zhuang et al., 1995). Therefore, water temperature relative to pulp temperature, disinfectant treatments, and other water quality constituents are jointly critical considerations for maintaining the safety and quality of the product.

The overall goal of this study was to evaluate the current operating standards for use of ClO_2 in postharvest washing of fresh tomatoes and develop data to support scientifically-based audit criteria. The specific objectives were: (1) conduct on-site assessment of ClO_2 dose management and quantitative microbiological water quality in commercial dump tank and flume systems, and (2) determine the comparative correlative capacity of ORP with other physicochemical parameters including pH, temperature and water turbidity to monitor, control and document water disinfection status within different commercial tomato packing operations.

7.2. MATERIALS AND METHODS

7.2.1. Tomato packing facilities

Cooperating tomato packinghouses in Florida (Facility A and B) and California (Facility C), United States of America, running with matched ClO_2 generation/injection systems for all water contact units were visited during their respective tomato production seasons in 2010. A total of 4 site visits in each State were performed randomly during the seasons. In general, the washing system of the three packinghouses consisted of a dump tank and a flume system and their respective re-circulation system tanks.

7.2.2. Water analysis

Water samples were taken from dump tank and flume system and their respective re-circulation system tanks every 30 min during each selected processing day (up to 6 hours per date). Water samples were taken to assess physicochemical parameters and to determine microbiological content. All measurements were repeated in triplicate.

7.2.2.1 Physicochemical parameters

Water samples of 500 mL were collected at each sample point to determine the oxidation reduction potential (ORP) (mV), ClO_2 concentration (mg/L) and turbidity (FAU) while pH, water temperature (°C), conductivity (S/cm) were directly measured at a consistent location of each tank and respective recirculation systems. The specifications of the instruments used in this study were as follows: Portable pH/temperature meter (Russell RL060P, Thermo Fisher Scientific Inc., Waltham, MA, USA) for temperature and pH; ORP-meter (Thermo Fisher Scientific Inc., Waltham, MA, USA) for ORP; portable colorimeters for turbidity (DR/850, Hach Company, Loveland, CO, USA) and ClO_2 residual (Pocket Colorimeter™ II, Hach Company, Loveland, CO, USA). Each physicochemical parameter, sampling point, and time was repeated three times.

7.2.2.2 Microbiological analysis

Standard enumeration methods were used to determine viable, culturable mesophilic bacteria suspended in water. Two random samples of 100 mL of water were collected from each sample point and immediately neutralized using 0.6% sodium thiosulphate ($Na_2S_2O_3$) (1N) or 100 µL ($Na_2S_2O_3$) (1N) in the FL and CA facilities respectively. The amount of $Na_2S_2O_3$ used was previously tested in the laboratory and was added in excess to ensure complete ClO_2 neutralization. Samples were immediately placed in refrigerated coolers and transported to respective and FL (CREC UF) and CA (UC Davis) laboratories where they were analyzed within 14 h of collection. Tenfold dilution series were prepared in 9 mL of sterile 0.1% buffered peptone water (BD

Diagnostics, Sparks, MD, USA). Total aerobic mesophilic bacteria (TAM) and total coliforms were enumerated from water samples. The following media and incubation conditions were used: plate count agar (PCA) (BD Diagnostics, Sparks, MD, USA) for TAM, incubated at 29ºC for 48 h and Chrom-ECC agar (Chrom Agar, Paris, France) for total coliforms and presumptive *E. coli*, incubated at 37ºC for 24 h. For trials 3 and 4 performed in CA, the population of total coliforms and *E. coli* was determined utilizing the QuantiTray™ Colilert® System (Idexx Laboratories Inc., Westbrook, Maine, USA). All microbial counts were reported as log colony forming units per 100 mL of sample (log CFU/100 mL).

7.2.3. Tomato analysis

Tomato samples were collected every 30 min during each selected processing day (5 – 6 h approximately) at each tomato facility. The sampling points were from the top and bottom of the field transport gondola (before washing) and at the end of the water contact before grading or worker contact (after spray brush bed washing).

7.2.3.1. Temperature

Five tomatoes were collected from each sampling point to determine on-site pulp temperature (ºC) of the fruit using a portable, calibrated temperature probe. The probe was calibrated in ice-water slurry before each sampling date. The temperature was measured for each tomato at the stem scar region (approx.1 cm deep).

7.2.3.2 Microbiological analysis

Two randomly collected samples of 5 tomatoes each were collected in sterile bags at each sampling point and immediately placed in refrigerated coolers, transported to respective CA and FL laboratories, stored at 2.5ºC, and processed within 14 h for microbiological analysis. Tomatoes were removed from storage and a 100 mL of sterile potassium phosphate buffer (3.9 mM KH_2PO_4; 6.1 mM K_2HPO_4) supplemented with 0.05% of Tween20 (Fisher, Fair Lawn, NJ, USA) were added. Tomatoes were vigorously rubbed by hand for 1 min to remove the attached bacteria from the tomato surface. Tenfold dilution series were prepared in 9 mL of sterile 0.1% buffered peptone water (BD Diagnostics, Sparks, MD, USA). TAM bacteria and total coliforms were recovered from the tomato samples. The following media and incubation conditions were used: PCA for TAM, incubated at 29ºC for 48 h and Chrom-ECC agar for total coliforms and presumptive *E. coli*, incubated at 37ºC for 24 h. All microbial counts were reported as log CFU/fruit.

7.2.4. Efficacy of the washing system on inoculated tomatoes

7.2.4.1. Bacterial strain and growth conditions

A *Pseudomonas fluorescens* (TVS074) strain, which is an antibiotic-resistant derivative strain to rifampicin (80 mg/L), was isolated via spontaneous mutation and used to facilitate detection and minimize interference with other bacteria during recovery. TVS074 is an EPA biological control formulation isolate registered by T. Suslow in 1994, after required EPA toxicological and non-target organism studies, and approved for commercial use on all fresh produce categories with no residue tolerance restrictions. *P. fluorescens* TVS074 was grown overnight on King's Medium B plates (0.15% $MgSO_4$ –Fisher, Fair Lawn NJ-, 1% Glycerol – Fisher, Fair Lawn NJ-, 2% K_2HPO_4 –EMD, Japan-, 2% Proteose peptone –BD Sparks, MD-, 15% Agar –BD Sparks, MD, USA) supplemented with 80 mg/L of rifampicin at 30°C. After incubation, cells were detached from the plates and re-suspended in Butterfield's buffer (Whatman Inc., Piscataway, NJ, USA). The cell suspension was washed twice by centrifugation at 4,000 rpm for 10 min and suspension in Butterfield's phosphate buffer. The final cell pellet was suspended in Butterfield's phosphate buffer and culture was adjusted to achieve a concentration of approximately log 9 CFU/mL. The final concentration was confirmed by plating on King's B plates supplemented with 80 mg/L of rifampicin.

7.2.4.2. Tomato inoculation

A total of 4 sets of 10 tomatoes were inoculated at room temperature by spot inoculation. The inoculated area was delimitated on the tomato surface. A total of 20 drops of 5 µL of inoculum (10^9 CFU/mL) were placed onto the surface. Inoculum was allowed to dry overnight at room temperature prior to processing. Inoculated tomatoes had an average concentration of log 8 CFU/fruit after inoculation.

7.2.4.3. Tomato processing

Each set of tomatoes was marked with a different color tape in order to differentiate them from the non-inoculated tomatoes and to facilitate the sample collection. Inoculated tomatoes were transported to Facility C and introduced to the system during standard washing operations and mixed with tomatoes from the field into the dump tank. Each set of inoculated fruit was introduced at different processing times with different water quality conditions, which were previously recorded. The tomatoes were collected after the final water contact and before grading and worker contact. Tomatoes were placed in sterile bags and stored in chilled coolers, transported to the laboratory and stored at 2.5°C. Tomatoes were analyzed within 14 h after collection for microbiological analysis. To determine the concentration of *P. fluorescens* TVS 074 on tomato surface an inoculated fruit not subjected to the commercial dump and wash system (n=10) was used as an internal control for survival. This set of tomatoes was

stored and transported under the same conditions as the tomatoes that were processed at the facility. This assessment was only performed in Facility C.

7.2.4.4. Bacterial enumeration

Tomatoes were individually placed in sterile bags (Whirl-Pak, Nasco, Modesto, California, USA) containing 50 mL of sterile potassium phosphate buffer (3.9 mM KH_2PO_4; 6.1 mM K_2HPO_4) supplemented with 0.05% of Tween20 (Fisher, Fair Lawn NJ, USA) and vigorously rubbed by hand. For *P. fluorescens* recovery, King's Medium B plates supplemented with 80 μg/L of rifampicin were used and were incubated at 30°C for 24 h. All microbial counts were reported as log CFU/fruit.

7.2.5. Contribution of washing water to the final tomato microbial load

7.2.5.1. Tomato surface sterilization and processing.

A total of 4 sets of 10 tomatoes each were submerged in a silver nitrate ($AgNO_3$) (0.1%) solution for 1 min at room temperature and rubbed and dried with sterile paper towels and ethanol (70% v/v) by hand for surface sterilization. Tomatoes were immediately introduced into the dump tank and collected, transported, and analyzed as previously described. This assessment was performed only in Facility C.

7.2.5.2. Bacterial enumeration

Populations of TAM and total coliforms were recovered from the tomato samples using the same methods previously described. As an internal control, surface sterilized fruit not subjected to the dump and flume wash system were used.

7.2.6. Statistical analysis

To establish temporal, water quality, and treatment differences between dump and flume tanks for all physicochemical parameters and bacterial populations, statistical analysis were performed using the MIX procedure, function of Statistical Analysis System (SAS Institute v. 9.2, Cary, NC, USA), mean separation was carried with Tukey's pair comparison. To determine differences in bacterial population between tomatoes collected from top and bottom of the harvest gondola a similar analysis was performed. When no significant changes were established during the time, all time points were averaged and comparison between dump and flume tank was carried out with a t-test paired comparison or one-way ANOVA to determine differences among facilities by using JMP ® (SAS Institute v.8). Statistical difference was established if p-values were smaller than 0.05. A correlation matrix among all physicochemical parameters and bacterial population was constructed using the CORR procedure, function of SAS which provided a value of Pearson correlation (R) and the p-value of the correlation. All data was evaluated for normality and homogeneity of variance using the UNIVARIATE procedure in SAS.

7.3. RESULTS

7.3.1. Water physicochemical conditions

Physicochemical parameters including pH, ORP, turbidity, conductivity and temperature in water utilized to wash tomatoes at three different facilities were assessed. For all trials and facilities no significant difference ($p>0.05$) was found between fruit contact tanks and their recirculation tanks, so values were averaged having a total of $n=6$ observations for both dump and flume tanks.

During each trial, physicochemical parameters during a regular processing day (up to 330 min) were monitored. Overall for all trials, ORP values were significantly higher in flume than in dump tanks ($p<0.05$), with the exception of Facility A during Trial 2 (Table 1). During the period of sampling on a given date, ORP values tended to remain constant for Facilities B and C, however for Facility A the ORP values of the flume tended to increase during the time course of daily operations (supplementary Table 1). For Facilities A and C, the dump tank ORP values were consistently measured at values below 600 mV, however in flume tanks the ORP values >600 mV were achieved over time. In contrast, Facility B maintained ORP values >600 mV in both tanks (Table 1). For Facility A during Trial 2 and Facility C during Trials 1 and 2, ORP values remained below the target values in both tanks (Table 1).

Conductivity and turbidity were the only parameters that showed a significant bivariate correlation ($p<0.05$) during the timeframe of each site visit. Both parameters increased during the sampling period (Table 1 and supplementary Tables 2 and 3) and were significantly larger ($p<0.05$) in dump tanks than in flume, with the exception of Facility B where both parameters were not significantly different between dump and flume tanks (Table 1). When comparing each of the trials, different conditions for each daily operation were recorded as conductivity and turbidity were significantly different between location and among trials (Table 1).

Turbidity values in flume tanks rarely exceeded values greater than 45 FAU. Following daily cleaning and complete fresh water addition at start-up, water turbidity was directly related to the incoming fruit load and condition which can carry varying amounts of soil and non-product organic matter. Several trials experienced accumulated turbidity values in the dump tank exceeding 300 FAU (Trials 2 and 4, Facility C), far greater levels as compared to the remaining trials (supplementary Table 3). In general, turbidity increased of the course of daily operations with occasional large spikes due to incoming loads (Table 1). For example, a change in turbidity from 264 to 320 FAU after 180 min (Trial 2, facility C) were observed in a tank of greater than 500 kL. In the same way, turbidity values from 316 to 594 FAU after 210 min were documented in Facility C during the fourth trial. Reductions in FAU values were periodically observed due to the periodic addition of partial clean water exchanges (approx. 30% turn-over) to the system by the facility operational staff. Although both conductivity and turbidity values

increased during sampling, ORP remained quite constant (Table 1 and supplementary Table 1).

Water temperature and pH were largely invariant during trials and did not have significant correlation with the time course of a daily event. Consequently their values for the entire trial at each location were averaged. FL facilities had no significant difference in pH and temperature between dump tank and flume system (Table 2). In contrast, Facility C had significantly higher pH and lower temperature in the dump tank than in the flume system (Table 2).

For all trials and locations, Pearson correlation among the different parameters, showed that ORP was negatively correlated with turbidity and positively correlated with temperature (Table 3). In contrast pH was not correlated ($p>0.05$) with ORP while conductivity was negatively correlated (Table 3).

To minimize water infiltration of fruit during packing, incoming tomatoes and processing water temperature should have a negative differential of at least 5.5°C; fruit pulp temperature being cooler than the water (Bartz, 1981, Ibarra-Sánchez et al., 2004; Zhuang et al., 1995). Water and incoming fruit temperature, as well as its temperature differential, were monitored throughout the site survey intervals for all trials. (Fig. 1 and 2). For Facilities A and B, temperature differentials less than 5.5°C were found in Trial A2 and B2. Particularly in Facility A during the second trial, the temperature differential was less than the target point for most part of the daily operations. For Facility C, there was a time period during trial 1 and 2 where this industry standard was not met.

7.3.2. Water microbiological quality

Population of TAM and total coliforms in water at each location during the operational monitoring time period were determined. For FL Facilities A and B, populations of TAM were not significantly different between dump and flume tanks ($p>0.05$) (Table 4A). In contrast, the population of TAM was always significantly lower in flume than in the dump tank system in the CA facility. Comparison among trials within each facility showed that the populations of TAM were similar within each location and a significant difference was established among the three tomato facilities (Table 4A). The general trend showed that population of TAM remained constant during the timeframe of a daily event (Table 4A). Pearson correlation analysis showed that ORP, pH, and temperature were negatively correlated with the population of TAM, while turbidity had a positive correlation (Table 3).

Populations of total coliforms were significantly greater in the dump tank than in the flume system ($p<0.05$) for all trials (Table 4B). Comparison among trials showed that population of total coliforms was similar in the dump tank for all tomato facilities. In contrast, more differences in population size were observed among trials in the flume

system (Table 4B). As observed with TAM, the population of total coliforms remained mostly constant during the each survey period (Table 4B). Additionally, a significant and positive correlation was established between total coliforms and turbidity, but a significant negative correlation occurred between the remaining physicochemical parameters monitored, with the exception of pH (Table 3). Detection of *E. coli* in dump tank was mostly negative for all trials in Florida and the first two trials in CA, although in the dump tank characteristic colonies on Chrom-ECC were sporadically detected. For Facility C, when detection method was changed to the QuantiTray™ Colilert® System for trials 3 and 4, to improve the sensitivity, *E. coli* was detected in the dump tank after 60 min of the first introduction of a tomato load. However, this bacterium was not detected in the flume tank at any sampling point (data not shown).

7.3.3. Washing effectiveness of dump tank and flume systems on tomato surface microbial populations

Tomato samples were collected from the top and bottom of the gondolas or bulk harvest bin for each trial during each operational day. No significant differences between the microbial populations from top and bottom of the collected tomatoes or between the different tomato lots arriving at the dump tank were found. Thus, these values were averaged by trial. Total microbial population and total coliforms did not change during the time of sampling intervals, and values were also averaged by trial.

In general, populations of TAM and total coliforms on tomatoes were not significantly different between tomatoes before and after washing ($p>0.05$). Microbial populations were greater after washing in 3 of the 8 trials for TAM and 6 of the 7 trials for total coliforms (Table 5A and B). Both groups of microbial populations tended to be greater in tomatoes from California (bush production) than from Florida (pole production).

To study the efficiency of the process in reducing the microbial load of challenge-inoculated tomatoes, *P. fluorescens* TVS 074 was applied to clean fruit at approx. log 8 CFU/fruit. During a regular operational shift, inoculated and marked tomatoes were introduced into the washing system with bulk, arriving fruit and collected at the end of the washing unit. Populations of *P. fluoresecens* TVS 074 was reduced by approximately 4-log units (Table 6). A set of tests were conducted to determine whether microbial loads would be acquired during washing. Three trials of surface sterilized tomatoes were processed during a regular packing shift. Populations of TAM increased from undetectable levels to approximately 3 log CFU/fruit after passing through the washing system. No significant difference was found among the three trials despite substantial variation in dump tank ORP values, and more uniform and seemingly compliant ORP levels in flume tanks (Table 7). Populations of TAM were below the limit of detection for surface sterilized tomatoes that were utilized as non-wash system contact control.

7.4. DISCUSSION

The implementation of practices that can minimize the risk of cross contamination with foodborne pathogens, especially whenever water contact is involved, is essential for a successful food safety program. Disinfection of water represents a vital management step to minimize transmission of pathogens within and between lots. Cross-contamination with plant pathogens has long been a concern for the fresh produce handling industry, including tomatoes, but in recent years *Salmonella* outbreaks associated with consumption of raw tomatoes have emphasized the importance to minimize risk to consumers (Bidol 2007; Greene 2005). In tomato packinghouses, large volumes of water during postharvest washing and handling are utilized. Water recirculation is a common practice in the industry due to economic considerations and wastewater discharge regulations, and thus effective re-conditioning by filtration and disinfection strategies are essential.

The use of ClO_2 is currently accepted within the guidelines for tomato good agricultural practices for postharvest water disinfection (FDACS, 2006). The FDA allows the use of ClO_2 as an antimicrobial agent in water used to wash fresh fruit and vegetables in a residual amount less than 3 mg/L. Also, this treatment must to be followed by a potable water rinse (CFR, 2007).

In this study, water treated with ClO_2 increases ORP, which has been established as a primary element to standardizing water disinfection monitoring, dose management, and documentation. ORP has been demonstrated to be a potentially useful measurement of the status of treated water to inactivate microorganisms, as well as an important component of a sound postharvest quality and safety program (Suslow, 2004). In this study ORP values were significantly ($p<0.05$) greater and more consistently maintained in the flume system than in dump tank for all tomato facilities (Table 1). ORP values of 650 to 700 mV are effective in inactivating spoilage bacteria and foodborne pathogens (*E. coli* and *Salmonella* spp.) within a few seconds, and spoilage yeast and fungi within a few minutes (Liao et al., 2007; Okull et al., 2006; Suslow 2001, 2004) in clean water systems. In general, these values were not achieved in the dump tank for all the facilities evaluated. However, adequate ORP values were seen in flume tanks except for Facility A (Table 1). ORP values were near 600 mV at the beginning of the operation in Facility C and throughout trial 2 in Facility B, but after the first load of tomatoes in the dump tank, values dropped to approximately 400 mV and remained constant during the time period of sampling evaluations (supplementary Table 1). This condition may represent a risk for cross contamination of produce if water disinfection efficiency cannot be reached in dump tank.

Turbidity and conductivity increased over time, most notably for turbidity levels (Table 1) due to incoming soil and organic matter associated with tomato harvests. Contrary to ORP behavior, turbidity was significantly greater in the dump tank than in flume water except for Facility B trial 2 (Table 1). This is supported by significant

Pearson correlation values ($p<0.05$) between ORP and turbidity. Similarly, populations of TAM and total coliforms were significantly less in the flume than in dump water (Table 4) and their populations were negatively correlated ($p<0.05$) with ORP but positively correlated with turbidity (Table 3). Conductivity was significantly greater in the dump tank thank in flume water for Facility C and Facility B trial 2.

The efficacy of ClO_2 for disinfection has been demonstrated in studies using regular tap water where the presence of organic matter has not been considered (Pao et al., 2007). Studies of disinfection efficiency of ClO_2 in wastewater have demonstrated that suspended solids and organic load have unfavorable effects in disinfection efficiency, mostly related with the ability of bacteria to attach to or derive protection from suspended organic matter (Ayyildiz et al., 2009; Narkis et al., 1994). In this study, the detection of *E. coli* in dump tanks but not in flume was observed. High values of turbidity were present in the dump tank, which presumably resulted in the inability of dump tank system to effectively inactivate *E. coli* cells in short retention times. Although ORP is considered as a primary indicator of oxidative disinfection for the inactivation of food-related pathogens at laboratory scale (Kim et al., 2000), our results suggest that reliance on monitoring ORP alone seems to be insufficient to predict microbial water quality within commercial tomato operations using ClO_2. Other physicochemical parameters should be taken into account to optimize microbial controls, particularly in the dump tank management.

Parameters such as pH and temperature remained mostly constant throughout operation, particularly in Facility C, and significant differences between dump tank and flume water for these conditions were found (Table 2). However both parameters were not significantly different ($p>0.05$) in Facilities A and B. Overall, pH was not correlated with ORP but it was negatively correlated with turbidity and population of TAM (Table 3). It is being suggested that oxidizing power of ClO_2 is not demonstrably pH-dependent within typical operating ranges, primarily because it does not hydrolyze in water to form hypochlorous acid (Beuchat, 1998). Additional studies demonstrated that bacteria like *E. coli* may be killed effectively with liquid ClO_2 in a pH-range from 3 to 8 in water (Huang et al., 1997).

Besides water disinfection treatment, water temperature can also impact the microbial safety and quality of tomatoes. Standard recommendations based on previous studies call for tomato dump tanks to be maintained at a temperature at leas 5.5 above than incoming fruit to prevent water infiltration and potential contamination with pathogens (Bartz, 1981, 1982, 1988; Ibarra-Sánchez et al., 2004; Zhuang et al., 1995). In these survey studies, water remained at a consistent, slightly elevated temperature, relative to ambient conditions, during the washing operation in all trials. However, interest was raised whether the measured differential between water and tomato pulp temperature met specifications and current Best Practice recommendations to limit water uptake by the fruit. Temperature differential varied among trials, but during time points of a daily shift was found to be less than the audit criteria of 5, which may

poses a risk to safety and quality. Variations among trials could be attributed to changes in environmental temperature during the day and harvesting conditions for each location. This parameter is considered a difficult focal point to control by the industry, particularly for different environmental and product conditions that can affect incoming product temperature. Thus, special attention in maintaining the temperature differential between water and tomato above 5.5°C must be taken into account to ensure food safety specifications without altering sensory attributes of the product. Overall results among trials varied for all parameters measured in water, presumably due to environmental and incoming fruit conditions as well as variances in daily operational management.

The efficiency of the washing system to reduce microbial load of the product acquired in the field is also an expected criteria for management focus. In this study populations of TAM and coliforms were not significantly different ($p>0.05$) or were significantly greater ($p<0.05$) between unwashed and washed tomatoes (Table 5). A previous study in a tomato facility at Charleston (South Carolina, USA) showed that total plate counts and *Enterobacteriaceae* counts on tomatoes were higher after washing with chlorine (90 – 140 mg/L) compared to unwashed tomatoes (Senter et al., 1985). Effectiveness of ClO_2 between 5 to 20 mg/L towards *S. enterica* and *E. carotovora* has been demonstrated in water although the sanitizing effects were reduced when tested on inoculated tomatoes; particularly if the inoculum suspension was allowed to substantially dry onto the tomato surface (Pao et al., 2007). It is likely the ClO_2 efficiency is reduced towards strongly attached microorganisms which may form protective aggregates over time (Iturriaga et al., 2007). In addition to washing operation through immersion in disinfected water, physical removal such as a spray brush-bed could improve microbial load reduction on tomato surfaces. The combination of physical removal with disinfected roller brushers and ClO_2 disinfected water immersion has been demonstrated to result in significantly greater *Salmonella* reduction on tomato surfaces than water immersion alone (Pao, 2009). Additional trials in Facility C showed that passage through the commercial washing operation achieved up to a 4 log reduction CFU/fruit of inoculated *P. fluorescens*. In contrast, surface sterilized tomatoes acquired approximately 4 log CFU/fruit of TAM, but not coliforms, during passage with incoming fruit loads through these washing operations.

The antimicrobial efficiency of ClO_2 in several commodities has been successfully demonstrated through laboratory scale studies (Gómez-López et al., 2009). However, the findings of this study indicate that ClO_2 can be an effective sanitizer for flume and spray-wash systems but current operational limitations greatly restrict performance in dump tank management. Under current conditions, the application of ClO_2 alone is unlikely to allow the fresh tomato industry to meet microbiological quality goals for dump tank management over typical commercial conditions. These findings suggest that water disinfection with ClO_2 should only be considered as part of an integrated strategy that successfully prevent contamination of the product by human or plant pathogens.

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TABLES AND FIGURES

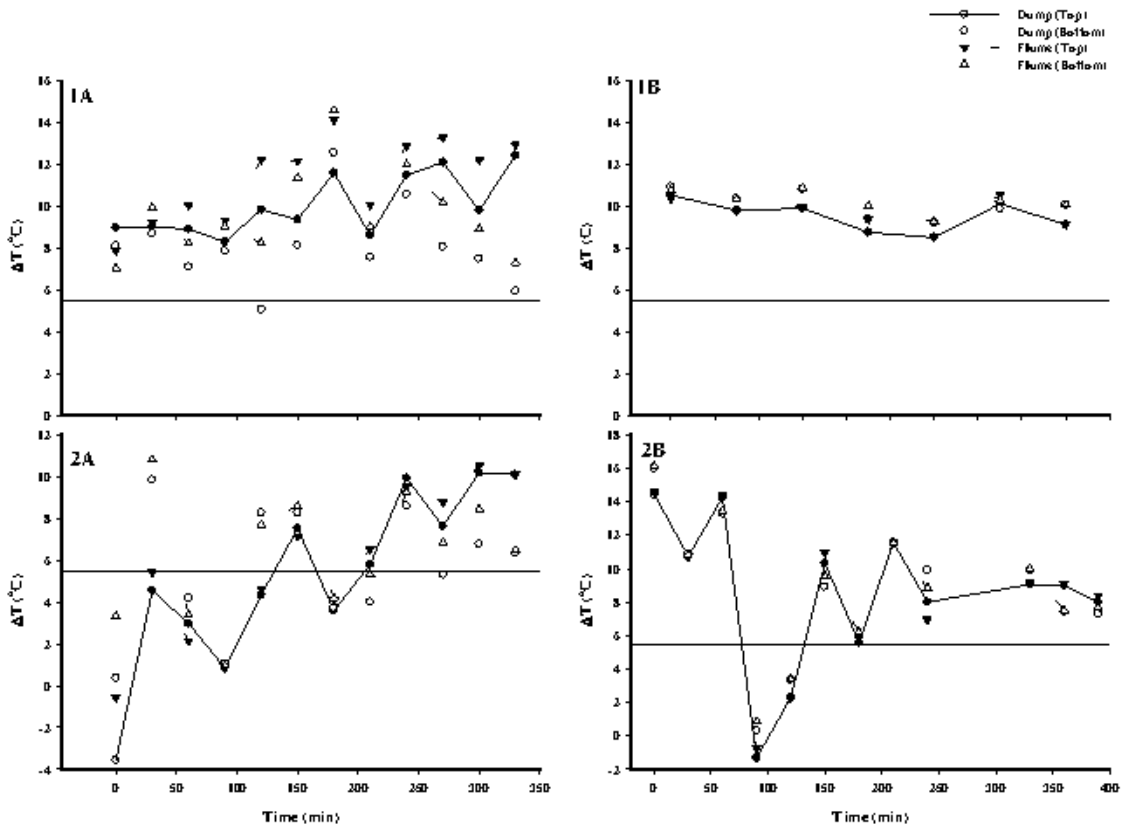

Figure 1. Tomato and dump water temperature differential record at the tomato Facility A (trials 1 and 2) and Facility B (trials 1 and 2) in Florida. Top and bottom refer to the sampling points on tomato gondola carrying incoming fruit. Temperature differential was calculated between incoming tomato and water temperatures. Horizontal solid line represents the minimum safe temperature differential (5.5°C) according to the Tomato Audit Protocol.

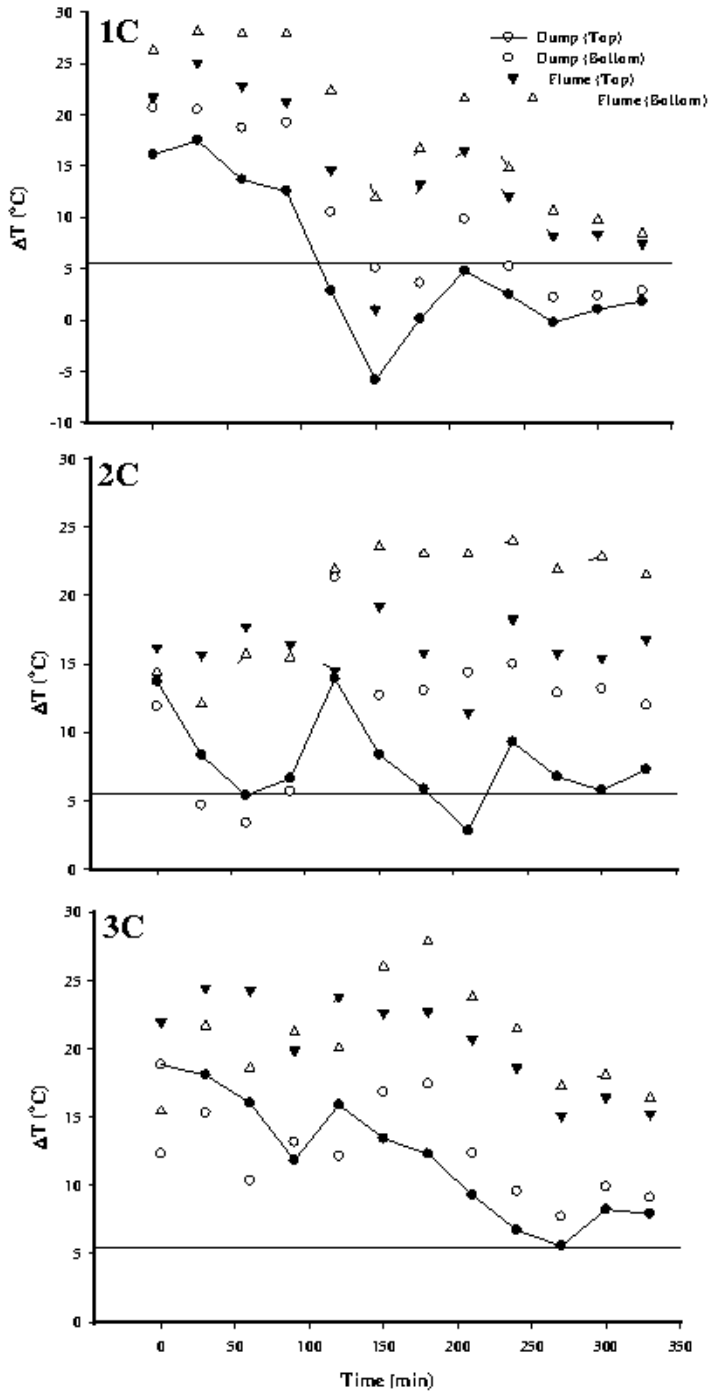

Figure 2. Tomato and dump water temperature differential record of the trials 1, 2 and 3 at the California tomato Facility C. Top and bottom refer to the sampling points on tomato gondola carrying incoming fruit. Temperature differential was calculated between incoming tomato and water temperatures. Horizontal solid line represents the minimum safe temperature differential (5.5°C) according to the Tomato Audit Protocol.

Table 1. ORP, turbidity and conductivity values in the tomato dump tank and flume system for facility and each operation day.

Facility		ORP (mV)[Z]		Conductivity (S/cm)[Z]		Turbidity (FAU)[Y]	
Florida	**Trial**	**Dump tank**	**Flume system**	**Dump tank**	**Flume system**	**Dump tank**	**Flume system**
A	1	427±106^{c,2}	530±110^{b,1}	1025±322^{d,1}	1240±160^{d,2}	2.33 – 172^{d,1}	1.66 - 40.0^{d,2}
	2	465±94.6^{b,c,2}	466±123^{c,1}	764±129^{e,1}	1090±107^{d,2}	4.50 – 131^{e,1}	3.00 - 45.0^{d,2}
B	1	537±147^{a,b,2}	637±67.0^{a,1}	1180±94.1^{c,d,1}	1216±177^{b,c,1}	2.00 – 57.0^{c,d,1}	2.33 – 53.3^{b,c,1}
	2	594±89.3^{a,2}	630±59.9^{a,1}	1476±289^{a,b,1}	1234±303^{a,1}	11.0 – 201^{a,b,1}	4.17 – 197^{a,1}
California							
C	1	437±33.7^{c,2}	488±83.0^{b,1}	1512±400^{a,1}	806±219^{b,2}	15.8 – 169^{a,1}	9.00 – 32.0^{b,2}
	2	430±90.2^{c,2}	518±117^{b,c,1}	1311±206^{c,1}	880±137^{c,2}	3.50 – 383^{c,1}	2.00 – 45.2^{c,2}
	3	431±146^{c,2}	669±12.5^{a,1}	1346±200^{b,c,2}	1187±99.3^{b,2}	0.16 – 305^{b,c,1}	0.66 – 42.3^{b,2}
	4	302±99.7^{d,2}	650±54.3^{a,1}	1147±166^{d,1}	1476±292^{b,1}	0.50 – 316^{d,1}	0.0– 110^{b,2}

[Z] Values represent the mean and standard deviation (n=3) for each trial during each operation day.
[Y] Values represent the range of turbidity from the beginning to the end of the operation time.
Different letters within each column denote significant difference among trials for all tomato facilities ($p<0.05$).
Different numbers between each row denote significant difference between dump tank and flume system ($p<0.05$).

Table 2. Water temperature and pH in the tomato dump tank and flume system for each facility and operation day.

Facility		pH		T (°C)	
Florida	**Trial**	**Dump tank**	**Flume system**	**Dump tank**	**Flume system**
A	1	$7.65\pm0.14^{b,1}$	$7.77\pm0.13^{b,c,1}$	$34.4\pm0.94^{c,2}$	$36.1\pm0.75^{c,d,1}$
	2	$7.66\pm0.17^{b,1}$	$7.65\pm0.19^{c,1}$	$34.9\pm1.42^{b,c,1}$	$35.7\pm0.61^{d,1}$
B	1	$7.36\pm0.11^{c,1}$	$7.41\pm0.15^{d,1}$	$36.8\pm0.27^{a,b,1}$	$36.9\pm0.32^{c,d,1}$
	2	$7.11\pm0.27^{d,1}$	$7.14\pm0.29^{e,1}$	$37.2\pm2.64^{a,1}$	$37.3\pm2.59^{c,1}$
California					
C	1	$7.32\pm0.19^{c,2}$	$7.83\pm0.16^{b,1}$	$34.2\pm2.45^{c,2}$	$42.3\pm1.76^{b,1}$
	2	$7.24\pm0.40^{c,d,2}$	$7.90\pm0.35^{b,1}$	$34.6\pm3.44^{b,c,2}$	$43.8\pm3.06^{a,1}$
	3	$7.94\pm0.41^{a,2}$	$8.21\pm0.45^{a,1}$	$33.9\pm4.89^{c,2}$	$42.1\pm4.13^{b,1}$
	4	$8.09\pm0.25^{a,1}$	$8.12\pm0.39^{a,1}$	$35.1\pm3.33^{b,c,2}$	$44.3\pm1.96^{a,1}$

Different letters within each column denote significant difference among trials for all tomato facilities ($p<0.05$).
Different numbers within each row denote significant difference between dump tank and flume system ($p<0.05$).

Table 3. Matrix of Pearson correlation values among different physicochemical water parameters and microbial populations.

Variables	pH	ORP	Turbidity	Conductivity	Temperature	TAM	Coliforms
pH	1	0.011	-0.305*	-0.483*	0.431*	-0.169*	-0.075
ORP		1	-0.409*	0.037	0.372*	-0.308*	-0.335*
Turbidity			1	0.416*	-0.439*	0.270*	0.166*
Conductivity				1	-0.054	0.032	-0.215*
Temperature					1	-0.531*	-0.446*
TAM						1	0.624*
Coliforms							1

* Denotes statistical significance of the correlation value ($p<0.05$)

Table 4. Comparison of bacterial population of total aerobic mesophiles (A) and total coliforms (B) between tomato dump and flume tank water.

Table 4A

Facility location	Trial	Dump	Flume	p-value[Y]
Florida		**log CFU/mL[Z]**		
A	1	4.01±0.36[b,c]	0.64±3.50[a]	0.0318
	2	3.44±0.98[c]	0.91±3.43[a]	0.8519
B	1	3.82±0.58[a,b,c]	1.21±3.34[a,b]	0.0052
	2	3.39±0.79[c]	0.70±1.64[a,b]	0.0579
California		**log CFU/mL**		
C	1	4.74±1.12[a]	2.22±0.74[b]	<0.0001
	2	4.68±1.35[a]	0.78±1.12[b]	<0.0001
	3	4.53±1.30[a,b]	2.44±2.86[c]	<0.0001
	4	ND	ND	

Table 4B

Facility location	Trial	Dump	Flume	p-value[Y]
Florida		**log CFU/100 mL[Z]**		
A	1	4.76±1.24[a,b]	3.50±2.28[a]	0.0009
	2	3.63±2.18[b,c]	3.43±1.97[a]	0.3418
B	1	5.00±0.69[a,b]	3.34±2.53[a]	0.0079
	2	4.24±2.80[a,b]	1.64±2.29[b,c]	0.0004
California		**log CFU/100 mL**		
C	1	2.85±2.07[c]	1.11±1.84[c,d]	<0.0001
	2	4.98±1.63[a]	0.17±0.71[d]	<0.0001
	3	2.69±1.31[c]	1.35±1.51[c]	<0.0001
	4	5.12±0.69[a]	2.78±1.03[a,b]	<0.0001

[Z] Values represent the mean and standard deviation.

[Y] P-value of the t-test comparison between bacterial populations of dump and flume tanks for each trial.

Different letter within each column denotes significant difference among trials for all tomato facilities.

(ND) Not determined for that trial.

Table 5. Comparison of bacterial population of total aerobic mesophiles (A) and total coliforms (B) on tomato surfaces between incoming tomatoes in the dump tank and tomatoes exiting the flume system.

Table 5A

Facility location	Trial	Before washing	After washing	p-value[Y]
Florida		**log CFU/fruit[Z]**		
A	1	4.98 ±1.03 ^{b,c}	4.67 ±0.70^{b}	0.922
	2	4.06 ± 1.05^{c,d}	4.38 ± 0.32^{b}	0.032
B	1	4.62 ± 0.65^{d}	4.39 ± 0.68^{b}	0.836
	2	4.85 ± 0.55 ^{b,c}	4.60 ± 0.85^{b}	0.902
California		**log CFU/fruit**		
C	1	5.18 ± 0.74^{b,c}	6.16± 0.45^{a}	<0.0001
	2	5.25 ± 0.83^{b}	6.13 ± 0.55^{a}	<0.0001
	3	6.53 ±0.97^{a}	5.50 ± 1.21^{a}	0.999
	4	ND	ND	

Table 5B

Facility location	Trial	Before washing	After washing	p-value[Y]
Florida		**log CFU/fruit[Z]**		
A	1	3.77 ± 1.11^{a,b}	3.35 ± 0.79^{c}	0.936
	2	3.18 ± 1.12^{b,c}	3.41 ± 0.62^{c}	0.148
B	1	3.12 ± 1.22^{b,c}	3.17 ± 0.54^{c}	0.427
	2	3.02 ± 1.34^{b,c}	3.39 ± 1.01^{c}	0.131
California		**log CFU/fruit**		
C	1	2.70 ± 1.39^{c}	4.85 ± 0.75^{a,b}	<0.0001
	2	3.80 ± 1.38^{a,b}	5.31 ± 0.65^{a}	<0.0001
	3	4.30 ± 1.59^{a}	4.44 ± 1.43^{b}	0.358
	4	ND	ND	

[Z] Values represent the mean and standard deviation.

[Y] P-value of the t-test comparison between bacterial populations of incoming unwashed tomatoes and after washing for each trial.

Different letter within each column denotes significant difference among trials for all tomato facilities.

(ND) Not determined for that trial.

Table 6. Reduction of *P. fluorescens* TVS 074 populations on tomato after processing through dump and flume tanks at tomato facilities.

	Dump	Flume		
Trial	**ORP (mV)**		**After washing log CFU/fruit[Z]**	**log reduction[Y]**
1	354	725	3.63±1.36	4.77
2	410	729	4.81±0.64	3.59
3	410	725	4.07±0.94	4.33
4	711	709	4.02±1.17	4.38

[Z] Values represent mean and standard deviation (n=10 tomatoes) after washing.
[Y] Value was calculated considering the average population of *P. fluoresences* (PTVS 074) on tomato surfaces that were not subjected to any washing treatment (~log 8 CFU/fruit)
Average temperature for three trials was 32.0±1.28 °C and 45.2±0.53 °C for dump and flume tanks respectively.
Average turbidity for three trials was 166±0.6 FAU and 21.3±7.4 FAU for dump and flume tanks respectively.

Table 7. Water contribution to the population of total aerobic mesophiles on surface sterilized tomatoes after processed in tomato dump and flume tanks.

	DUMP TANK	FLUME	Population of TAM[Z]
Trial	**ORP (mV)**		**log CFU/fruit**
1	470	802	3.84±0.36
2	825	787	3.61±0.52
3	779	790	2.84±0.21

[Z] Values represent mean and standard deviation (n=10 tomatoes)
Average temperature for three trials was 28.2±12.5 °C and 40.6±0.37 °C for dump and flume tanks respectively.
Average turbidity for three trials was 325±39.7 FAU and 55.0±9.64 FAU for dump and flume tanks respectively.
Total coliforms were not detected on tomatoes for any of the three trials (detection limit log 2 CFU/ fruit)

SUPLEMENTARY MATERIAL

Supplementary table 1. Comparison of oxidation reduction potential in water between dump tank and flume system

DUMP TANK								
Facility location	**Florida**				**California**			
	A		**B**		**C**			
TRIAL[Y]	**1**	**2**	**2**	**1**	**2**	**3**	**4**	
time	**ORP (mV)**				**ORP (mV)**			
0	272±33.3	392±31.8	407±15.7	364±15.5	427±29.8	680±13.9	689±12.7	548±64.9
30	487±22.6	411±34.4	371±10.5	685±1.53	362±±37.6	340±32.0	702±7.11	249±29.1
60	358±7.23	394±24.8	341±3.21	504±15.8	433±18.8	347±44.6	642±35.9	285±16.2
90	314±26.9	468±44.9	627±2.64	679±2.31	435±31.8	379±69.5	309±18.9	198±6.93
120	532±176	497±116	666±0.57	573±18.3	452±25.1	373±33.5	335±23.9	284±13.8
150	440±5.46	454±55.4	673±0.00	666±1.00	464±18.7	389±31.4	368±20.8	272±11.2
180	439±27.4	518±63.9	670±1.00	652±1.15	410±7.03	420±25.1	344±11.3	262±11.9
210	458±16.4	610±18.5	ND	635±2.52	455±15.7	434±12.1	364±12.4	290±14.6
240	451±1.51	428±3.83	ND	654±1.00	457±11.9	438±6.98	353±14.1	315±19.5
270	472±2.06	398±64.3	ND	627±1.53	434±13.6	44.7±14.3	352±10.8	ND
300	449±13.6	499±135	ND	561±2.08	457±11.5	455±6.79	341±4.31	ND
330	444±6.57	505±148	ND	555±2.08	453±16.7	452±5.16	370±8.47	ND
FLUME SYSTEM								
	A		**B**		**C**			
TRIAL[Y]	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**4**
time	**ORP (mV)**				**ORP (mV)**			
0	470±43.0	395±81.6	553±20.5	621±36.7	458±21.1	668±8.95	665±23.7	582±43.5
30	520±18.6	569±142	562±93.1	692±5.39	590±4.51	604.11.1	687±5.67	568±55.5
60	393±9.81	502±44.3	637±35.4	681±10.6	602±3.67	548±29.1	683±2.81	659±23.3
90	333±1.64	519±102	645±44.5	691±1.17	616±3.62	321±63.6	667±7.06	622±13.9
120	681±32.2	373±52.3	684±7.50	6569±116	586±40.3	355±42.7	658±5.78	674±1.33
150	406±2.99	369±36.9	688±7.14	655±11.9	425±965	387±63.9	656±1.52	696±1.21
180	599±24.6	480±159	688±2.66	594±46.1	392±10.9	444±102	650±1.17	695±1.17
210	469±12.93	494±172	ND	648±1.87	455±13.8	548±35.1	665±0.55	697±0.52
240	607±7.95	459±159	ND	664±3.68	457±16.2	548±57.6	670±1.51	688±0.55
270	634±10.25	400±51.8	ND	624±1.94	407±19.0	587±22.6	667±0.52	ND
300	621±11.6	510±111	ND	560±3.45	425±11.7	604±15.6	673±1.05	ND
330	616±6.86	518±134	ND	554±2.81	438±15.1	593±26.6	677±3.33	ND

Data represents mean ± standard deviation for each time point and tomato facility
(ND) Not determined for that time point

Supplementary table 2. Comparison of conductivity in water between dump tank and flume system

DUMP TANK								
Facility location	**Florida**				**California**			
	A		**B**		**C**			
TRIAL[¥]	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**4**
time	**Conductivity (S/cm)**				**Conductivity (S/cm)**			
0	601±9.68	782±44.2	1053±2.08	1093±5.23	883±16.3	922±11.7	976±5.16	916±12.1
30	696±23.8	780±1.00	1086±2.08	1114±2.52	930±10.9	1012±4.08	1172±7.52	953±16.3
60	724±3.82	645±1.53	1128±2.88	1211±2.52	1083±8.17	1113±12.1	1185±8.37	1015±15.2
90	775±7.44	628±0.57	1170±3.61	1234±2.00	1173±20.6	1195±5.47	1182±7.53	1053±13.6
120	842±34.7	855±1.73	1230±7.521	1343±6.11	1315±5.47	1268±7.52	1203±5.16	1217±15.06
150	855±9.48	924±0.00	1271±4.73	1388±11.02	1607±13.66	1335±5.47	1337±13.66	1277±8.16
180	994±16.3	684±0.00	1322±15.5	1468±4.93	1667±30.7	1370±20.0	1370±6.34	1352±11.7
210	1262±325	661±0.57	ND	1536±3.05	1760±0.00	1438±18.4	1423±8.16	1393±26.6
240	1479±5.09	851±141	ND	1697±6.55	1812±24.83	1460±0.00	1490±8.94	1143±16.3
270	1507±17.7	955±4.11	ND	1791±5.13	1892±14.7	1500±0.00	1543±5.16	ND
300	1313±13.3	674±33.2	ND	1919±1.15	1985±12.3	1550±6.32	1620±0.00	ND
330	1246±73.2	646±57.8	ND	ND	2032±27.8	1572±4.08	1647±5.16	ND
FLUME SYSTEM								
	A		**B**		**C**			
TRIAL[¥]	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**4**
time	**Conductivity (S/cm)**				**Conductivity (S/cm)**			
0	594±7.33	1021±0.57	1059±13.7	1098±8.93	900±20.9	922±4.08	980±0.00	906±8.17
30	609±3.67	912±0.00	1085±7.82	1119±4.14	1022±24.8	945±5.47	1040±0.00	990±14.1
60	614±1.67	727±0.57	1119±7.28	1176±8.45	1090±20.0	967±8.98	1045±5.47	1057±18.6
90	631±3.88	712±0.57	1183±12.3	1236±4.07	1173±23.4	998±4.02	1045±5.48	1098±16.0
120	665±10.3	988±0.58	1237±14.1	1327±20.0	1203±16.3	10454±8.36	1072±4.08	1220±20.9
150	659±5.15	1143±0.00	1292±13.7	1386±11.6	1310±15.5	1097±5.16	1172±11.7	1125±487
180	690±2.28	782±0.57	1334±13.4	1485±8.05	1310±14.1	1123±5.16	1228±4.08	1452±23.1
210	1164±59.9	748±0.57	ND	1543±7.98	1322±13.3	1165±5.47	1292±4.08	1547±22.5
240	1200±20.6	965±1.00	ND	1689±14.5	1348±17.2	1182±4.08	1348±19.4	1713±86.6
270	967±10.3	991±1.53	ND	1796±13.3	1362±14.7	1200±6.32	1405±8.37	ND
300	952±84.4	788±0.58	ND	1927±5.65	1400±6.33	1217±8.17	1463±12.11	ND
330	926±076.7	784±0.58	ND	ND	1437±15.1	1222±4.08	1505±5.47	ND

Data represents mean ± standard deviation for each time point and tomato facility
(ND) Not determined for that time point

Supplementary table 3. Comparison of turbidity in water between dump tank and flume system

DUMP TANK								
Facility location	**Florida**				**California**			
	A		**B**		**C**			
TRIAL[¥]	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**4**
time	**Turbidity (FAU)**				**Turbidity (FAU)**			
0	2.33±2.33	4.50±1.22	2.00±1.00	11.0±2.00	15.8±10.2	3.5±1.38	0.16±0.41	0.50±1.23
30	23.3±2.06	22.8±1.83	12.3±2.88	13.0±1.00	46.2±7.78	44.5±2.07	21.5±4.85	34.5±2.88
60	48.7±2.66	32.0±1.67	17.0±1.73	25.6±2.32	72.7±4.92	83.2±3.19	44.3±4.18	224±12.1
90	77.5±5.64	45.8±6.62	41.3±2.08	37.7±3.05	97.8±8.06	106±2.25	66.6±1.12	503±4.23
120	80.5±2.43	53.7±4.37	50.7±1.53	54.6±3.22	118±7.81	179±5.75	78.0±6.06	539±2.50
150	85.8±3.31	59.8±5.46	53.0±2.00	59.7±0.57	139±8.80	258±6.31	78.3±4.13	635±28.8
180	117±11.9	72.06.600	57.0±3.61	64.0±1.00	143±15.4	320±49.3	107±7.78	661±8.67
210	114±5.12	91.3±1.51	ND	76.7±2.31	142±7.18	264±3.98	163±9.39	594±11.2
240	128±10.2	101±3.19	ND	95.7±4.93	148±6.12	283±1.75	227±6.00	316±18.6
270	136±8.91	109±2.88	ND	128±2.88	153±8.66	354±12.3	268±9.13	ND
300	148±6.34	116±3.78	ND	165±3.05	160±2.66	382±8.91	292±3.19	ND
330	172±13.8	131±5.09	ND	201±6.55	169±9.31	383±13.8	305±6.81	ND
FLUME SYSTEM								
	A		**B**		**C**			
TRIAL[¥]	**1**	**2**	**1**	**2**	**1**	**2**	**3**	**4**
time	**Turbidity (FAU)**				**Turbidity (FAU)**			
0	1.66±1.03	3.00±1.41	2.33±2.25	4.17±1.60	9.00±5.96	2.00±1.89	0.66±1.21	0.00
30	2.00±2.28	6.83±3.54	6.83±1.94	11.2±1.17	10.7±3.33	4.50±2.66	6.00±2.28	0.00
60	9.17±3.43	6.50±1.64	10.7±2.25	22.2±3.31	14.0±5.67	8.33±2.65	8.33±2.25	22.8±0.75
90	15.3±3.01	8.67±1.21	33.7±1.75	22.2±2.53	14.02.175	14.5±1.38	8.00±2.09	50.5±2.81
120	14.3±1.75	16.5±1.76	49.0±1.41	49.7±3.78	19.3±0.95	23.2±2.64	7.17±1.72	75.5±4.04
150	17.3±1.86	19.8±2.04	51.0±1.67	57.8±2.86	23.0±1.26	31.7±1.63	8.66±1.03	8172±3.09
180	23.2±2.40	21.2±2.32	53.3±3.27	66.8±3.97	22.2±2.85	39.5±3.94	11.2±1.60	95.7±3.09
210	28.3±1.21	30.5±1.22	ND	74.8±3.13	27.8±5.25	35.2±4.66	18.0±1.55	92.8±4.57
240	32.0±3.16	30.8±1.60	ND	88.5±4.42	30.2±2.48	38.7±1.03	29.3±3.26	110±2.63
270	34.2±2.31	33.7±1.75	ND	127±3.01	29.5±1.05	44.0±5.69	32.3±2.42	ND
300	36.2±1.47	40.8±1.94	ND	165±2.4	33.7±5.68	45.17±6.88	38.0±4.05	ND
330	40.0±1.67	45.0±2.76	ND	197±4.72	32.0±1.55	45.17±1.33	42.3±2.34	ND

Data represents mean ± standard deviation for each time point and tomato facility
(ND) Not determined for that time point

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CHAPTER 8

Chlorine dioxide dose, water quality and temperature affect the oxidative status of tomato processing water and its ability to inactivate *Salmonella*

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8.1. INTRODUCTION

Consumption of raw fresh tomatoes has been frequently associated with outbreaks of human salmonellosis over the past four decades (FDACS, 2006). Diverse *Salmonella enterica* serotypes have been implicated in multiple cases of illness and outbreaks, including several large multi-state episodes with hundreds of clinical cases, including Newport, Typhimurium, Javiana, Anatum, Thompson and Muenchen (CDC 2005, 2007). In order to minimize the microbiological hazards associated with whole fresh and fresh-cut tomato products the United Fresh Produce Association (United Fresh) in collaboration with the North American Tomato Trade Work Group published the 'Commodity Specific Food Safety Guidelines for the Fresh Tomato Supply Chain' (United Fresh, 2008). Most recently, in 2010, United Fresh posted the collaborative effort to develop *Food Safety Programs and Auditing Protocol for the Fresh Tomato Supply Chain* (Tomato Audit Protocol) which includes standards for postharvest water quality management and minimum process control criteria for antimicrobial dose (United Fresh, 2010).

Washing fresh produce with clean water can reduce potential contamination associated with the crop and non-food materials (e.g. soil, leaf trash, and other foreign materials) often co-accumulated in harvest containers. However wash and product handling water, such as in dump tanks and flumes, may also be a source of contamination or a major point of cross-contamination. Due to regional water shortage, increasing cost of assuring potable water quality for food contact, and regulated waste water treatments and handling, reconditioning and reuse of postharvest water is a common practice and recommended by the United States Department of Agriculture (USDA, 1999). Large volumes of re-circulated water are commonly used in packinghouses for tomato postharvest handling operations. The re-circulated water systems, including dump tanks, flumes, and re-packing systems often lead to accumulation of organic matter and can potentially become a vehicle of cross-contamination for incoming fresh product (Ongeng *et al*., 2006). In particular, the wash water can easily become laden with pathogens from contaminated raw tomatoes and spread throughout the supply chain. Dump tank and flume water have received particular attention is as a potential source of enteric pathogen contamination or cross contamination during processing operations (FDACS, 2006). Left untreated, water intended to clean produce functions for a brief period early in a shift but contaminates produce later in the daily operation (Brackett, 1992). Moreover, for the specific case of the tomato postharvest handling, an inadequate washing management program may result in the infiltration of water to the interior of fruit and cause microorganism internalization (Bartz, 1982).

The addition of antimicrobial agents to recycled water can inactivate bacterial cells and fungal conidia or spores in water, helping minimize cross-contamination. As fresh market tomatoes are commonly consumed raw, disinfection in the washing and packing phase constitute an available and practical means of risk reduction.

Chlorination is the most commonly sanitizing technique used to minimize the microbiological hazards associated in the fresh plant produce industry (Artés *et al.*, 2009). However, some problems have long been recognized related to its use, such as potentially hazardous disinfection-by-products formation, its strong pH dependence for rapid antimicrobial action, and the potential for gas emission that may affect workers comfort and safety (Ölmez and Kretzschmar, 2009). Other approved postharvest water sanitizers include peroxiacetic acid, aqueous ClO_2 and ozone. Due to the above mentioned issues, efforts to identify and evaluate alternative sanitizing agents to chlorine has become of increasing concern and priority for various industry sectors. The U.S. Food and Drug Administration (FDA) approved formulations of ClO_2 as an antimicrobial agent in water used to wash fresh fruit and vegetables in a residual amount less than 3 mg/L. Also, this treatment must to be followed by a potable water rinse (CFR, 2007). Based on its molecular weight and its ability to accept electrons, ClO_2 has approximately 263% available chlorine, which is more than 2.5 times the oxidizing capacity of hypochlorous acid (Hass, 1990; Suslow, 2004).

The use of ClO_2 as a water treatment aide for minimally processed produce has been recently reviewed (Gómez-López *et al.*, 2009). However, there is limited research available about its antimicrobial effect on plant processing water and the level of restriction of cross contamination potential. Beuchat *et al.* (2001) suggested a standard approach to test and contrast the effectiveness of antimicrobials in killing microorganisms during postharvest management of raw fruit and vegetables. In order to develop a standard methodology several factors including type of produce, type of pathogen, inoculum type and size, and conditions for storing produce between the time of application of inoculum and treatment with disinfectant were posed. A limitation to adoption within commercial systems revolves around the fact that most research related to the evaluation of the antimicrobial effectiveness in reduction of pathogens during washing are conducted solely under laboratory scale studies, that used clean tap water or other optimal water constituent conditions, that do not anticipate the full range of industrial scenarios. In particular, the bactericidal effect of ClO_2 towards human pathogens has been studied in model systems that only partially mimic typical commercial system conditions with variations of temperature and levels of organic matter that could affect efficiency of a given disinfectant (Junli *et al*, 1997). When evaluating a sanitizer, water quality parameters such as pH, temperature, turbidity, conductivity, organic matter content should be considered, in order to avoid the generation of data under unrealistic conditions with limited capacity to translate into practical applications (Gil *et al.*, 2009). Presence of organic loads in water can provide protection to bacteria through stabilization of the cell membranes and restricting access of a sanitizing agent to key cellular components for inactivation (Virto *et al.*, 2005). Microorganisms attached or embedded in particles have been shown to demonstrate an apparent increased resistance to inactivation by chlorine compared to non-attached microorganisms (LeChevallier *et al.*, 1984; Dietrich *et al.*, 2003; Bohrerova and Linden, 2006). Cross contamination with *E. coli* between inoculated and non inoculated fresh-cut escarole washed with different water quality was impacted by microbial and organic

load present in re-circulation water (Allende *et al.*, 2008). During produce washing, it has been demonstrated that once cross-contamination occurs, further washing with disinfectant solutions, including ClO_2, were ineffective in complete control of the attached contamination (López-Galvez *et al.*, 2010; Nou *et al.*, 2011)

Previous assessment at different tomato packinghouses in California, by the research lead, documented fluctuations in physicochemical water conditions including temperature, turbidity, conductivity and pH during multiple daily operational surveys. Turbidity can vary from 0 to 300 FAU, or greater, and from 0 to 50 FAU for dump tank and flume systems, respectively, during a regular processing day. Moreover, processing water temperature in dump tank and flume can reach 40ºC, or higher, to avoid water infiltration into tomatoes (Unpublished results).

The objectives of the current work were to (1) evaluate the effect of ClO_2 concentration, water temperature and turbidity on the system control-point ORP levels and (2) assay the effect of ClO_2 on the survival of several *S. enterica* serotypes in fresh tomato processing water under different temperatures and water quality conditions.

8.2. MATERIALS AND METHODS

8.2.1. Synthetic water preparation

Synthetic tomato processing water was designed to reproduce a consensus processed water composition based on previous water assessment at tomato packing houses (Unpublished results). To simulate the background oxidative demand of processing water, tomato plants with adhering soil were taken from University of California (Davis, CA) research farm and submerged in water. Dilutions were prepared an adjusted to create the following turbidity values: 0, 22, 43 and 160 FAU. Synthetic water was autoclaved to minimize interference with other bacteria and to facilitate enumeration of inoculated *Salmonella.*

8.2.2. Bacterial strains and growth conditions

Seven *S. enterica* serotypes including Poona (PTVS026), Garminara (PTVS041), Michigan (PTVS042), Enteriditis (PTVS044), Agona (PTVS043), Montevideo (PTVS045) and Newport (PTVS077) were used. An antibiotic-resistant derivative strain to rifampicin (80 mg/L) was isolated for each isolate via spontaneous mutation and used to minimize interference with other bacteria and to facilitate the detection and recovery for each serotype. *S. enterica* was grown overnight in 9 mL of tryptic soy broth (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin at 37°C. After incubation, cultures were centrifuged at 4,000 rpm for 10 min. The pellet was re-suspended and washed twice with Butterfield's phosphate buffer (Whatman Inc., Piscataway, NJ, USA). The final cell pellet was suspended in Butterfield's phosphate buffer to make an initial cell density of approximately 10^9 CFU/mL. The final concentration was confirmed by plating on Tryptic Soy Agar (TSA)

(BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin (TSA-rif).

8.2.3. Experiment design, detection and recovery of *Salmonella enterica*

A factorial combination of the following conditions was evaluated: water turbidity (0, 22, 43 and 160 FAU), water temperature (10, 25 and 40°C), and ClO_2 concentration (1, 3 and 5 mg/L). For each condition, *S. enterica* sv. Newport cells were added to 100 mL of synthetic water to achieve a final concentration of 10^7 CFU/mL. After homogenous distribution of the inoculum, ClO_2 was added to reach 1, 3 or 5 mg/L with continuous stirring. Immediately, an aliquot of 1 mL of sample was taken and dispensed in a tube containing 9 mL of Dey/Engley (DE) neutralizing broth (BD Diagnostics, Sparks, MD, USA) supplemented with 80 mg/L of rifampicin to inactivate residual ClO_2 at the end of treatment contact times; 5, 10, 15, 30, 45, 60, 75, 90 and 120 s. Tenfold dilutions were prepared in 9 mL of sterile 0.1 % buffered peptone water (BD Diagnostics, Sparks, MD, USA). Samples were plated on TSA-rif and incubated at 37°C for 24 h to determine the log reduction of *S. enterica* sv. Newport for each treatment interval. Additionally, tubes were incubated at 37°C for 24 h to establish an enrichment-based presence/absence test for samples anticipated to be below the limit of detection for direct enumeration. Enrichment allowed a qualitative determination of the contact time needed for complete inactivation of *S. enterica* sv. Newport. These experiments were compared to a standard disinfection using NaClO at 3 concentration levels (5, 25 and 50 mg/L; pH = 7) at 25°C. For the remaining serotypes, an enrichment-based qualitative test, as described above, was used. All the turbidity and ClO_2 conditions were tested at 25°C. All analyses were made in triplicate.

8.2.4. Physicochemical analysis

Changes in the ORP (mV), pH, and residual ClO_2 concentration (mg/L) were monitored in a separate test without pathogen inoculation, for biosafety considerations, and using the same factorial experimental design (water temperature = 10, 25 and 40°C; Turbidity: 0, 22, 43 and 160 FAU). The ORP, pH and residual ClO_2 concentration were measured in a system containing 100 mL of synthetic water and after the addition of ClO_2 for 2 min as described above. All physicochemical parameters were determined using standard protocols. Specifications of the instruments used are listed below: portable pH/temperature meter (Russell RL060P, Thermo Fisher Scientific Inc., Waltham, MA, USA) for temperature and pH; ORP-meter (Thermo Fisher Scientific Inc., Waltham, MA, USA) for ORP; portable colorimeters for turbidity (DR/850, Hach Company, Loveland, CO, USA) and ClO_2 residual (Pocket Colorimeter™ II, Hach Company, Loveland, CO, USA). The samples regarding 160 FAU water were pre-filtered by using a 0.45 µm filter before measuring the ClO_2 residual to avoid interference from suspended solids and the ClO_2 colorimeter. All analyses were made in triplicate.

8.2.5. Statistical design

The experiment was based on a 3x3x3 factorial design: turbidity with three levels: 0, 22, 43 and 160 FAU, x temperature with three levels10, 25 and 40ºC and ClO_2 concentration with three levels 1, 3 and 5 mg/L. The resulting data was analyzed using Statistical Analyses System (SAS) 9.2. (SAS Institute, Cary, NC, USA), with analysis of variance (ANOVA). A correlation matrix among all physicochemical parameters and bacterial population was constructed using CORRELATION procedure function of SAS which provided a value of Pearson correlation (R) and the p-value of the correlation. For all comparisons, a significant difference was established when $p<0.05$. All data was previously evaluated for normality and homogeneity of variance using the UNIVARIATE procedure function of SAS.

8.3. RESULTS

8.3.1 Effect of temperature and turbidity on *Salmonella enterica* survival in water treated with different ClO_2 concentrations

Water temperature had a pronounced effect on the efficiency of ClO_2 to inactivate *S. enterica* sv. Newport. An increase in water temperature led to greater log reduction of the pathogen at all ClO_2 concentrations tested (Fig. 1). Under water conditions of 25 and 40ºC, an approximately 7-log reduction was achieved within 30 s when ClO_2 concentration was 3 or 5 mg/L (Fig. 1A and B). However at a water temperature of 10ºC, or lower, for a ClO_2 concentration of 1 mg/L, the reduction of *Salmonella* viability was approximately 4-log (Fig. 1C). In contrast to the temperature effect, increases in water turbidity decreased the log reduction of *S. enterica* (Fig. 2). This tendency was more evident for 1 and 3 mg/L ClO_2 (Fig. 2 B and C), however when the ClO_2 concentration was 5 mg/L, the log reduction was not significantly different regardless of turbidity conditions within the test system. Statistical analysis of the main effects on the population of *S. enterica* showed a significant effect of the three parameters: temperature, turbidity and ClO_2 concentration (Table 1). Additionally, significant interactions between turbidity and both, temperature and ClO_2 concentration were found but such interactions were not apparent between ClO_2 concentration and temperature (Table 1).

8.3.2 Effect of temperature and turbidity on the contact time needed for inactivation of *Salmonella enterica* in water treated with different ClO_2 concentrations

During each disinfection performance assessment experiment, temporal sample aliquots were further enriched to determine total inactivation of *S. enterica* within a 2 min period (Table 2). As in the enumerative survival experiments, contact times for inactivation were of shorter periodicity at higher temperature and ClO_2 concentration but were extended as higher values of water turbidity were introduced (Table 2). For

water turbidity of 160 FAU, 1 and 3 mg/L ClO_2 were not effective in inactivating *S. enterica* sv. Newport regardless of the water temperature. A 5 mg ClO_2 /L concentration was sufficient to inactivate *S. enterica* in water (160 FAU) at 25 and 40ºC after 75 and 120 s, respectively. For water temperature of 10ºC, ClO_2 levels of 1 and 3 mg/L were unable to completely inactivate freely suspended *Salmonella* cells (Table 2).

Comparisons between ClO_2 and NaClO, for their ability to inactivate *S. enterica* cells were determined. For NaClO, lesser contact times were needed to inactivate approximately 7-log CFU/mL of *Salmonella* in a range of 5 to 50 mg/L at 25ºC. When ClO_2 was utilized (Table 3), *S. enterica* populations dropped within the first 5 s to a recoverable population below the limit of detection, except when water turbidity increased to 160 FAU (data not shown).

8.3.3 Comparison of the susceptibility of different *Salmonella enterica* strains to ClO_2 under different conditions of water temperature and turbidity

In general, heterogeneous inactivation times for the different serotypes regarding turbidity and ClO_2 concentration were observed (Table 4). A ClO_2 dose of 1 mg/L was not able to inactive any strain within 120 s regardless of the water turbidity. A ClO_2 concentration of 3 mg/L effectively inactivated the serotypes Newport, Gaminara, Poona and Enteriditis after 30, 60, 75 and 120 s, respectively, for a turbidity value of 43 FAU but it was unable to inactivate *S.* Montevideo and Michigan serotypes. However, 3 mg/L ClO_2 was not able to inactivate any *Salmonella* serotype at 160 FAU. Differences among *Salmonella* serotypes, turbidity and ClO_2 concentrations on the inactivation time were found. All serotypes were inactivated in timeframes less than 120 s by using 5 mg/L ClO_2 at the 43 FAU condition. However, at 160 FAU, only the Newport serotype was inactivated within 120 s. Consequently *Salmonella* serotypes could be ranked according to their resistance to the ClO_2 doses tested as follows: Newport < Gaminara < Poona < Enteriditis < Agona < Montevideo < Michigan, in order of increasing tolerance of the oxidative disinfectant conditions employed in this study.

8.3.4 Effect of water turbidity, temperature and ClO_2 concentration on the oxidation reduction potential

Water temperature and turbidity in the model system affected ORP values. ClO_2 concentrations of 3 and 5 mg/L were sufficient to maintain ORP values greater than 650 mV when water turbidity was adjusted at 22 and 43 FAU (Fig. 3A and B). However, when water turbidity was 160 FAU, ORP values tended to decrease steadily throughout the experimental period time and failed to exceed 500 mV. When 1 mg/L ClO_2 was used at 22 FAU water turbidity, ORP values greater than 650 mV were achieved. Nevertheless, for water adjusted to 43 or 160 FAU, ORP tended to decrease or failed to achieve an oxidative status greater than 300 mV (Fig. 3C).

Under conditions held at 5 mg/L ClO_2 and 160 FAU turbidity, ORP was observed to achieve values greater than 650 mV within 10 s. However, ORP tended to decrease when the water temperature was 25 or 40°C, but remained relatively constant when temperature was held at 10°C (data not shown). For 1 and 3 mg/L ClO_2 ORP values were not significantly different whichever water temperature was tested, reaching values lower than 600 mV (data not shown). For water turbidity of 22 and 43 FAU, ORP values in excess of 650 mV were reached regardless temperature conditions (data not shown).

Residual ClO_2 concentrations were monitored after addition during 2 min. For the three ClO_2 concentrations assayed, after 5 s of their addition a lowering in the residual ClO_2 concentration was documented. Lower loses of ClO_2 were observed at 10°C than at 40°C as well as at 22 FAU than at 160 FAU (Table 5).

From the statistical analysis on the effect of the main factors studied in the model system, it was shown that ORP was significantly affected ($p<0.05$) by water temperature, turbidity and ClO_2 concentration (Table 1). Interactions among factors were significant between turbidity and both, temperature and ClO_2 concentration, but not between temperature and ClO_2 concentration (Table 1). Changes in pH and residual ClO_2 throughout time were monitored, but not significant effect associated to different conditions of temperature, turbidity or ClO_2 concentration was found (Table 1).

8.3.5 Relationship between water physicochemical parameters and inactivation of Salmonella enterica.

Pearson correlation (R) among all physicochemical parameters and the inactivation of *S. enterica* was determined (Table 6). Population of *S. enterica* in disinfected water was negatively correlated ($p<0.05$) to contact time, temperature, ORP and ClO_2 concentration but positively correlated to turbidity (Table 6). ORP was positively correlated to contact time, ClO_2 concentration and pH, but negatively correlated to water temperature and turbidity.

8.4. DISCUSSION

Water turbidity and temperature and ClO_2 concentration showed a significant interacting effect and were well correlated with the survival potential of *S. enterica* and in the associated ORP of the simulated process water (Tables 1 and 6). Contact times of 30 s were sufficient to provide a ~7-log reduction of the pathogen in a range of 25 to 40°C, 3-5 mg/L ClO_2 and water turbidity of 0 to 40 FAU (Fig. 1 and 2) These water quality conditions are often present in commercial flume tanks handling fresh market tomatoes. However when *S. enterica* cells were exposed to lower water temperature (10 °C), high values of water turbidity (160 FAU) or lower ClO_2 concentration (1 mg/L), the survival and the contact time needed for inactivation significantly increased and conditions were often insufficient to eliminate the pathogen (Tables 2 and 4). These

findings were correlated with the ORP, where higher values of turbidity limited the ability of the water system to maintain values of ORP higher than 650 (Fig. 3), which are often specified for an adequate bactericidal effect in industry guidance standards (United Fresh, 2008, 2010).

Although the interaction between ClO_2 and organic matter does not produce toxic by-products, as occurs with NaClO, ClO_2 is able to oxidize a large fraction of natural organic matter (Swietlik *et al.*, 2004). Thus, it is likely that oxidation of organic matter diminish its ability to increase and maintain the oxidation status of the processing water (Fig. 3). The unfavorable influences of organic matter load in terms of chemical oxygen demand (COD) for the disinfection efficacy of ClO_2 in wastewater have been reported. The inactivation of *E. coli* in artificial wastewater decreased as the COD increased at the same ClO_2 concentration (Ayyildiz *et al.*, 2009). In addition, suspended solids in solution have a protective role in limiting microorganism inactivation by ClO_2 due to entrapment of the bacteria within organic matrix or through bacterial aggregation in water, which enhances protection against diverse disinfectant reagents (Narkis *et al.*, 1995; Barbeau *et al*, 2005). Similar effects associated with organic load in the processing water have been reported for other water disinfection strategies such as acidic electrolyzed water, where its bactericidal effect towards *E. coli* O157:H7, *S. enterica* sv. Typhimurium and *Listeria monocytogenes* and spoilage microorganisms was reduced with an increase in water organic demand (Ongeng *et al.*, 2006; Park *et al.*, 2009). It is recommended that chlorinated water should not reach turbidity values greater than 300 FAU, after which, it should be replenished with clean water (Suslow, 2004). However this study indicates that ineffectiveness of the disinfectant solution could also occur with lower levels of turbidity, and often dump tanks in fresh tomato handling industry can surpass these values. Consequently the control of presence of large amounts of organic matter and total suspended solids appears to be a key step in an adequate washing management program with ClO_2.

Temperature is an important variable impacting the activity of oxidant-based disinfectants (Barbeau *et al.*, 2005). Elevated water temperatures and disinfectant doses and contact times generally favor the inactivation of microorganisms by ClO_2 (Barbeau *et al.*, 2005; Son *et al.*, 2005). The effect of temperature in this study showed that an increase in temperature reduced the contact time needed for *Salmonella* inactivation (Fig. 1). This effect was previously demonstrated for the inactivation of *E. coli* in tap water, in a range of 5 to 35°C, where reduction to levels below the limit of detection varied from 35 to 7 s respectively (Benarde *et al.*, 1967). In this study, at 10°C water temperature, the ORP reached and maintained higher values than when water temperature was 25 or 40 °C, for conditions when ClO_2 dose was set at 5mg/L (data not shown). Additionally, lower ClO_2 residual was achieved at 10°C compared to 40°C (Table 5). The ORP often correlates well with the antimicrobial potential of the water and, with certain dose range limitations, is directly correlated with the concentration of the oxidant (Robbs *et al.*, 1995). Previous studies showed that ClO_2 residual in hot water was lower than in cold water, presumptively related with faster solubilization of

ClO_2 (Zhang *et al.*, 2008). Moreover, other studies suggest that in solution, ClO_2 gas quickly equilibrate in the vessel headspace. In the case of dump tanks and open flumes, the airspace is infinite and will presumably reduce the amount of ClO_2 to zero (Mahovic, 2002) which can be exacerbated with an increase in temperature and exposure to sunlight. It is likely that although low temperature could maintain higher ORP values by preventing ClO_2 losses, an increase in temperature can promote better dissolution of ClO_2 in water, thus increasing its oxidative action favoring inactivation of *Salmonella* cells. It is important to recognize that it is imperative to monitor ClO_2 and ORP levels, to ensure adequate disinfection, particularly in open systems as dump tanks and open flumes for tomato washing.

Simulating commercial conditions for fresh tomato processing water were considered in this study to establish the variability of the parameters evaluated. Flume tanks, based on our experience in monitoring several operations, will rarely exceed turbidity values higher than 40-50 FAU, and although temperature can affect residual levels of ClO_2 in water, results indicate that the ClO_2 was effective in reducing pathogens levels exceeding a 6-log parameter. In contrast, on-site surveillance at a tomato packinghouse established that dump tanks can reach high turbidity levels during daily operations and thus current permissible regulatory levels of ClO_2 addition might not be sufficient to successfully prevent cross contamination of plant and human pathogens.

Efficiency of ClO_2 to disinfect dump tanks and flume systems for tomato processing water should be evaluated in a context that considers factors that can impair its effectiveness. Therefore, implementation of any water disinfection strategy should consider parameters of disinfectant concentration, water temperature, suspended solids and organic load to ensure that produce washing can act as a point of control to minimize risk of contamination with human and plant pathogens during production of fresh horticultural produce.

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TABLES AND FIGURES

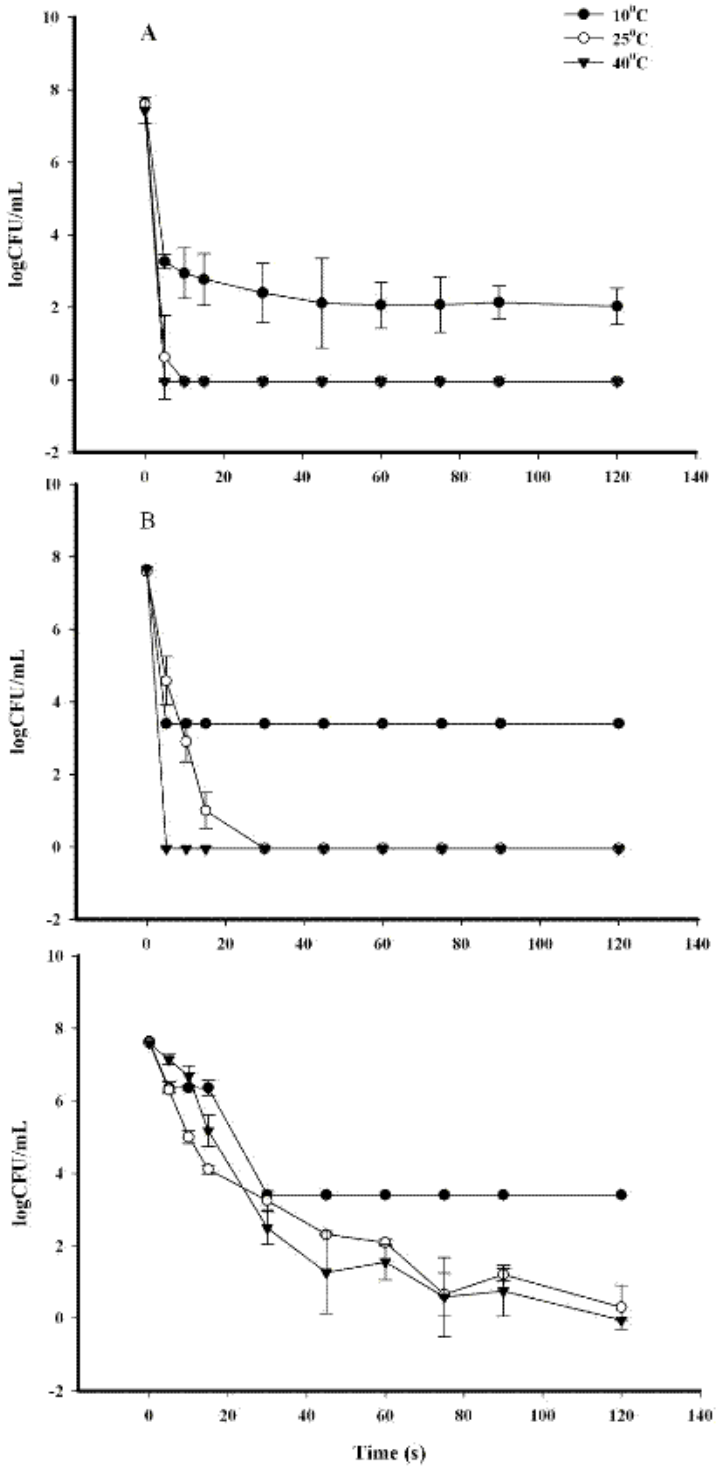

Figure 1. Effect of temperature on the survival of *S. enterica* by sanitizing with (A) 5 mg/L, (B) 3 mg/L, (C) 1 mg/L ClO_2 within 2 min of contact time at 43 FAU water turbidity

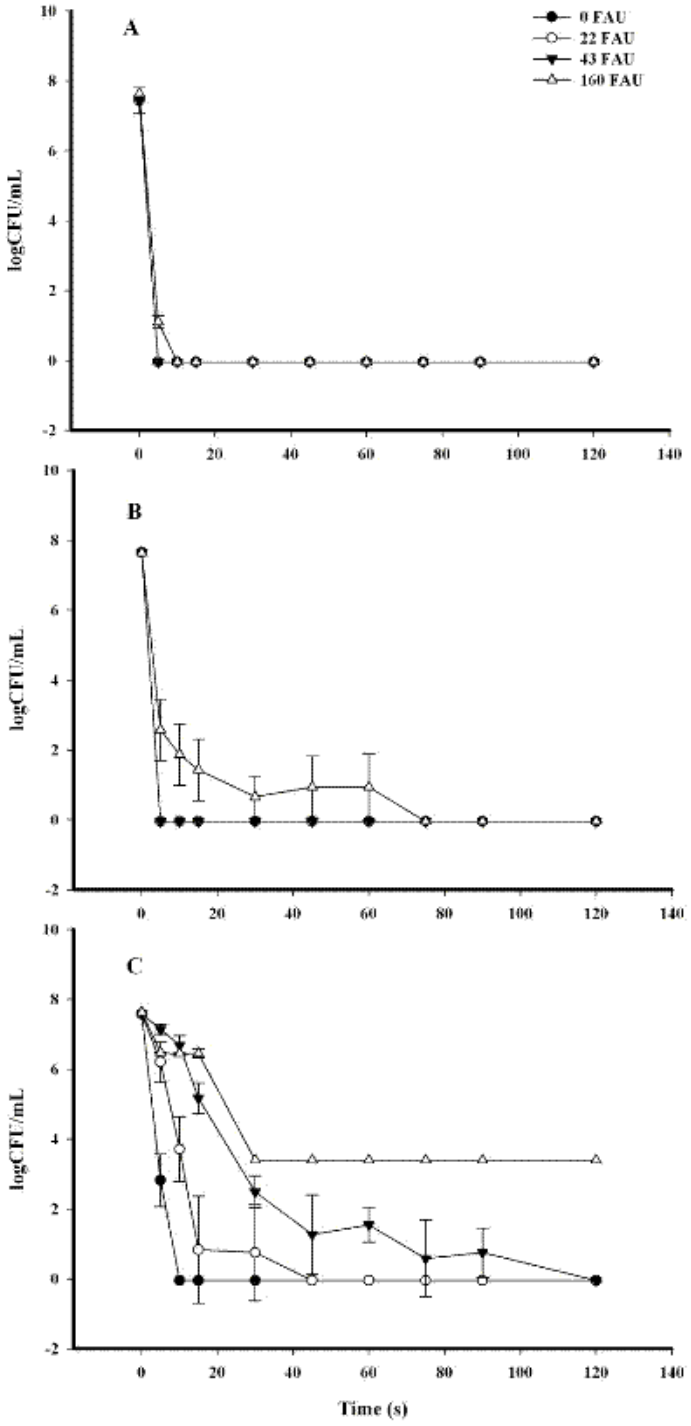

Figure 2. Effect of turbidity on the survival of *S. enterica* by sanitizing with (A) 5 mg/L, (B) 3 mg/L, (C) 1 mg/L ClO_2 within 2 min of contact time at 40°C water temperature.

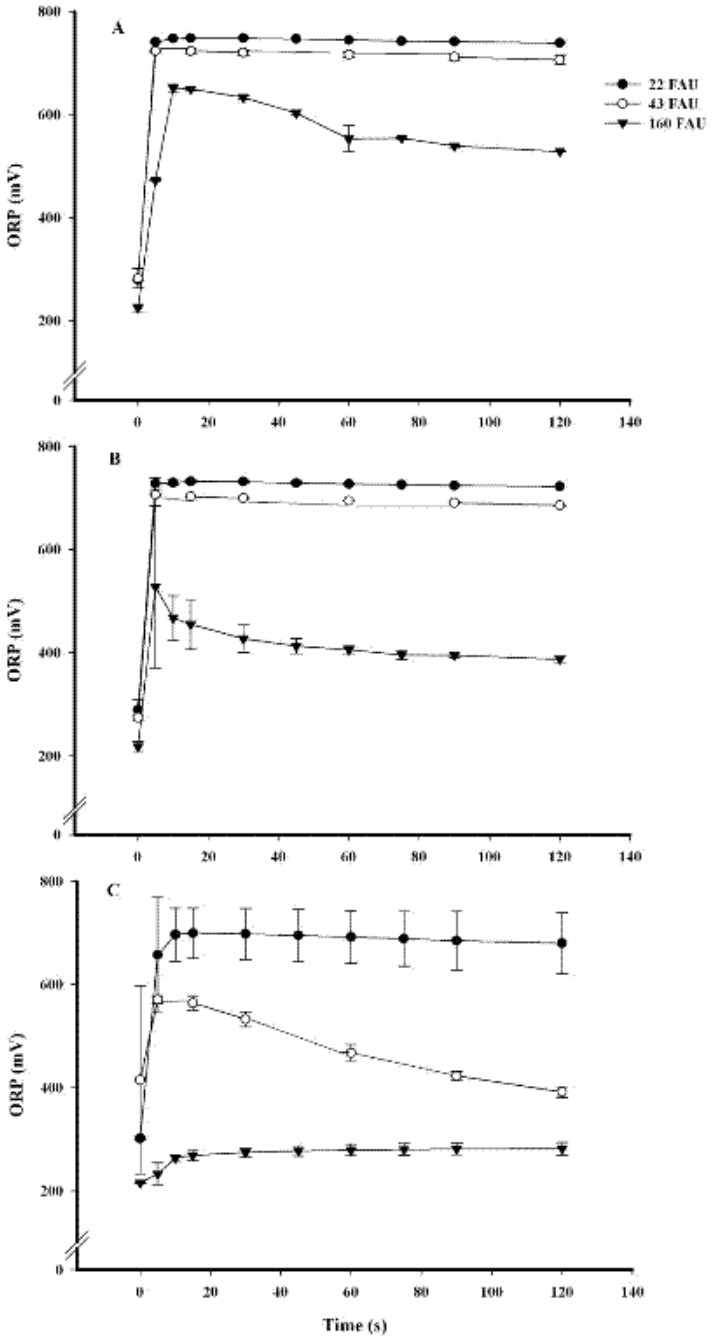

Figure 3. Effect of turbidity on the ORP with (A) 5 mg/L, (B) 3 mg/L, (C) 1 mg/L ClO_2 within 2 min of contact time at 40℃ water temperature.

Table 1. Analysis of variance of population of *S. enterica*, ORP, pH and ClO_2 residual as influenced by water temperature (T), turbidity (F) and ClO_2 concentration ($[ClO_2]$)[a].

Source of variation[a]	df	Population of Salmonella		ORP		pH		ClO_2 residual	
		F value	p-value	F value	p-value	F value	p-value	F value	p-value
T	2	14.8	<0.0001	6.1	0.0024	0.8	0.4241	0.7	0.5216
F	2	5.8	0.0006	212.1	<0.0001	1.5	0.2281	1.2	0.3118
T x F	4	6.9	<0.0001	2.5	0.0392	0.9	0.4734	0.7	0.5987
$[ClO_2]$	2	45.4	<0.001	78.9	<0.0001	0.8	0.4591	2.1	0.1299
T x $[ClO_2]$	4	1.7	0.1488	0.2	0.9364	0.9	0.4866	0.7	0.5797
F X$[ClO_2]$	4	2.9	0.008	16.5	<0.0001	0.9	0.443	0.8	0.556
TxFx$[ClO_2]$	8	1.1	0.3392	0.7	<0.6930	0.9	0.4883	0.8	0.6211

[a] Temperature (10, 25 and 40°C), Turbidity (22, 43 and 160 FAU), ClO_2 concentration (1, 3 and 5 mg/L) and initial population of *S. enterica* of ~ 7 log CFU/mL.

Table 2. Effect of ClO_2 concentration, water temperature (T) and turbidity on the contact time (s) needed for inactivation of *S. enterica* sv. Newport.

Turbidity (FAU)	T (°C)	1 mg ClO_2/L			3 mg ClO_2/L			5 mg ClO_2/L		
		10	25	40	10	25	40	10	25	40
0		ND	60	5	ND	10	<5	ND	10	<5
22		>120[a]	90	45	>120	15	5	45	5	<5
43		>120	>120	90	>120	30	5	>120	5	<5
160		>120	>120	>120	>120	>120	>120	>120	120	75

[a] Contact time (s) needed for the inactivation of *S. enterica* sv. Newport. Initial concentration was ~7 log CFU/mL.
ND: Not determined

Table 3. Effect of NaClO concentration (mg/L) and water turbidity (FAU) on the contact time needed (s) for inactivation of *S. enterica* sv. Newport.

	NaClO (mg/L)		
Water turbidity (FAU)	5	25	50
0	5[a]	<5	<5
43	120	15	<5
60	>120	75	15

[a] Contact time (s). For all conditions plate counts were recorded. Except for 160 FAU and 5 mg/L NaClO, all treatments had counts lower than the limit of detection (0.4 log CFU/mL) after 5 s of NaClO addition

Table 4. Comparison of the contact time (s) needed for inactivation of different *S. enterica* serovars under different water turbidity (FAU) and ClO_2 concentrations (mg/L) at 25°C of water temperature.

Salmonella strain	**Newport**	**Gaminara**	**Poona**	**Enteritidis**	**Agona**	**Montevideo**	**Michigan**
Turbidity (FAU)	*1 mg ClO_2/L*						
42	>120[a]	>120	>120	>120	>120	>120	>120
160	>120	>120	>120	>120	>120	>120	>120
	3 mg ClO_2/L						
42	30	60	75	120	>120	>120	>120
160	>120	>120	>120	>120	>120	>120	>120
	5 mg ClO_2/L						
42	5	30	60	90	90	120	120
160	120	>120	>120	>120	>120	>120	>120

[a] Contact time (s). Initial concentration of *S. enterica* culture was ~7 log CFU/mL

Table 5. Effect of water turbidity (FAU) and water temperature (°C) on the residual ClO_2 concentration (mg/L) during time.

ClO_2 (mg/L) [a]	**1**			**3**			**5**		
T (°C)	**10**	**25**	**40**	**10**	**25**	**40**	**10**	**25**	**40**
	Water turbidity 22 FAU								
Time (s)									
5	0.67[b]	0.66	0.55	3.3	2.57	2.24	4.61	3.99	3.54
15	0.69	0.64	0.5	3.14	2.45	2.12	4.39	3.77	3.40
60	0.73	0.60	0.51	2.91	2.25	1.91	4.32	3.62	3.31
120	0.67	0.57	0.48	3.04	2.21	1.97	4.20	3.61	3.08
	Water turbidity 43 FAU								
Time (s)									
5	0.37	1.01	1.06	2.87	3.19	3.06	4.05	3.94	4.43
15	0.28	1.05	1.03	2.11	3.24	3.04	3.87	3.9	4.65
60	0.20	1.00	0.95	1.96	3.00	2.79	3.62	3.77	3.97
120	0.19	0.94	0.88	2.01	2.85	2.28	3.66	3.40	3.33
	Water turbidity 160 FAU								
Time (s)									
5	0.49	0.43	0.43	1.92	1.13	1.35	1.58	1.93	0.26
60	0.50	0.46	0.36	1.16	0.99	0.97	1.76	1.50	0.21
120	0.50	0.32	0.39	1.17	0.96	0.84	1.59	1.42	0.23

[a] Initial ClO_2 concentration at time t=0s.
[b] Residual ClO_2 concentration (mg/L).

Table 6. Correlation matrix among physicochemical parameters and inactivation of *S. enterica* with ClO_2.

	Contact time	**Water temperature**	**Turbidity**	**ClO_2**	**Salmonella population**	**ORP**	**pH**	**ClO_2 residual**
Contact time	1[a]	0	0	0	-0.529***	0.195***	-0.0612	-0.055
Water temperature		1	0	0	-0.147***	-0.111*	-0.0513	-0.07
Turbidity			1	0	0.064*	-0.609***	0.076	-0.078
ClO_2				1	-0.311***	0.370***	-0.021	0.095
Salmonella population					1	-0.603***	0.063	-0.058
ORP						1	-0.112*	0.069
pH							1	-0.008
ClO_2 residual								1

[a] Values represent Pearson correlation (R).
(*, **,***) Denotes significance of the correlation at $p<0.05$, 0.001, 0.0001 respectively

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CONCLUSSIONS

The general conclusions of the current Doctoral Thesis are as follows:

1. High O_2 MAP showed a beneficial effect by lowering the natural microflora growth of FC Red Chard up to 7 days at 5ºC compared to the NaClO disinfected control under passive MAP. He-enriched MAP showed a beneficial effect in retaining the total chlorophyll content throughout shelf life. Such results suggest that He and O_2-enriched MAPs are both efficient tools for keeping overall quality of FC Red Chard better than a conventional NaClO disinfected passive MAP.

2. Washing with EW seems to be a promising disinfection technique that appears to be as effective as NaClO on FC Mizuna. Both AEW and NEW showed an inhibitory effect on natural microflora growth compared to a control washing with deionised water. EW treatments assayed did not affect the surface structure of the Mizuna leaves after washing and maintained the quality attributes throughout 11 days at 5ºC. The effects of NEW and AEW on by-product formation, as well as on processing equipments, should be further studied.

3. Low to moderate doses of ASC (100-500 mg/L) showed an initial antimicrobial efficacy on epiphytic microflora and *E. coli* that was as effective as NaClO regardless of the contact time on FC Tatsoi baby leaves. ASC treatment might damage the superficial vegetable tissues and could promote microbiological growth. Therefore, the effects of ASC on surface damage to the leaves, on nutritional quality of Tatsoi baby leaves and on the processing equipment should be further investigated.

4. Selected levels of ClO_2 and NaClO were unable to fully disinfect the applied *Salmonella* and *E. coli* O157:H7 from inoculated Red Chard. While ClO_2 substantially prevented *E. coli* O157:H7 cross contamination, it was not effective for the *Salmonella* isolate. Water from a centrifugation-dewatering step represents a potential risk of cross-contamination to products as well as to equipment used immediately prior to packaging. These results further suggest that the centrifugation effluent water could be used as a potential sample point to evaluate lot contamination and cross-contamination, in the processing chain, by low levels of pathogens that are undetectable by conventional sampling methods.

5. The fate of *E. coli* and *E. coli* O157:H7 during production, harvest, minimal processing and storage-distribution of FC Mizuna, Tatsoi and Red Chard baby leaves are reported in the current Thesis. The ability low of levels of *E. coli* to survive during production, after disinfection and chilling storage of has been demonstrated. These findings provide useful data to further develop an adequate science-based risk assessment during the production and minimal processing of these crops.

6. UV-C pretreatment alone or combined with superatmospheric O_2 packaging successfully controlled TAM and psychrophilic bacteria growth on FC Tatsoi. These innovative sustainable treatments can be useful tools to the industry for keeping quality and safety of the FC Tatsoi baby leaves and while minimizing water consumption and wastewater discharge rates. Scientific data about the effects of innovative MAP on overall quality of FC baby leaves is scarce and relatively unclear. In this way, the current experiments improve the lack of information regarding the effect of high O_2 active MAP combined with UV-C pre-treatment on overall quality parameters of FC Tatsoi baby leaves.

7. ClO_2 efficacy is strongly affected by water quality. Passage through a commercial tomato washing operation achieved up to a 5 log reduction CFU/fruit of inoculated *P. fluorescens*. In contrast, surface sterilized tomatoes acquired approximately 4 log CFU/fruit of TAM during passage with incoming fruit loads through these washing operations. This study provides in-factory data that supports the use of ClO_2 as an effective sanitizer for flume and spray-wash system; however, current operational limitations greatly restrict performance in dump tank management. ClO_2 alone is unlikely to allow the fresh tomato industry to meet microbiological quality goals for dump tank management over typical commercial conditions.

8. Increases in water turbidity reduce ORP and increase the contact time required to inactivate *S. enterica* in synthetic processing water treated with ClO_2. An increase in temperature and ClO_2 concentration reduces the contact time and achieves a 6 log reduction of *S. enterica* within a 2 minutes time frame. However, water composition strongly affects the ORP values, which can limit total inactivation of *S. enterica*. Generated data demonstrated the effect of water quality and temperature to maintain an effective ORP towards inactivation of *S. enterica*, which could impact the current definition of adequate water quality and safety standards.

An effective sanitation program cannot solely rely on the application of a sanitizer or a combination of disinfection treatments for keeping quality and safety of fresh and FC vegetables. This requires that more effort be placed on the development of a well integrated production, handling, processing and distribution system. Specific attention should be directed on keeping appropriate chilling storage temperatures, optimal packaging and distribution conditions throughout the entire commercial life. In addition, GAP, GMP and HACCP programs should be implemented in order to minimize the risk of contamination and to assure safety and overall quality of produce for consumers.

SCIENTIFIC PUBLICATIONS FROM THE DOCTORAL THESIS

Book chapter

- Artés, F., Gómez, P.A., **Tomás-Callejas, A.**, Artés-Hernández, F., 2011. Sanitation of fresh-cut fruit and vegetables: New trends, methods and impacts. In: McMann, J.M. (Ed), Potable water and sanitation. Nova Science Publishers, Hauppauge, New York, USA. pp. 1-36.

Original papers published in peer-reviewed journals (ISI, Institute for Scientific Information)

- Artés-Hernández, F., Robles, P.A., Gómez, P.A., **Tomás-Callejas, A.**, Artés, F., 2010. Low UV-C illumination for keeping overall quality of fresh-cut watermelon. *Postharvest Biology and Technology 55*, 114-120.

- **Tomás-Callejas, A.**, Martínez-Hernández, G.B., Artés, F., Artés-Hernández, F., 2011 Neutral and acidic electrolyzed water as emergent sanitizers for fresh-cut Mizuna baby leaves. *Postharvest Biology and Technology 59*, 298-306.

- **Tomás-Callejas, A.**, Boluda, M., Robles, P.A., Artés, F., Artés-Hernández, F., 2011. Innovative active modified atmosphere packaging improves overall quality of fresh-cut Red Chard baby leaves. *LWT Food Science and Technology 44*, 1422-1428.

Original papers published in Acta Horticulturae

- Robles, P.A., **Tomás-Callejas, A.**, Escalona, V.H., Artés, F., Artés-Hernández, F., 2010. High helium controlled atmosphere storage decreases microbial growth and preserves quality on fresh-cut Mizuna baby leaves. *Acta Horticulturae 877*, 663-668.

- **Tomás-Callejas, A.**, Boluda, M., Robles, P.A., Artés, F., Artés-Hernández, F., 2010. Vitamin C and chlorophylls retention on minimally fresh processed Red Chard baby leaves packed under non-conventional modified atmosphere. *Acta Horticulturae 877*, 707-714.

Original papers submitted to peer-reviewed journals (ISI, Institute for Scientific Information)

- **Tomás-Callejas, A.**, López-Velasco, G., Artés, F., Artés-Hernández, F. Optimization of acidified sodium chlorite to improve quality of fresh-cut Tatsoi baby leaves. Submitted, March 2011.

- **Tomás-Callejas, A.**, Otón, M., Artés, F., Artés-Hernández, F. Combined effect of UV-C pretreatment and high oxygen packaging for keeping quality of fresh-cut Tatsoi baby leaves. Submitted, March 2011.

- **Tomás-Callejas, A.**, López-Gálvez, F., Sbodio, A., Artés, F., Artés-Hernández, F., Suslow, T.V. Chlorine dioxide and chlorine effectiveness to prevent *Escherichia coli* O157:H7 and *Salmonella* cross-contamination on fresh-cut Red Chard.Submitted, April 2011.

- **Tomás-Callejas, A.**, López-Velasco, G., Camacho, A.B., Artés, F., Artés-Hernández, F., Suslow, T.V. Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions. Submitted, Abril 2011.

- **Tomás-Callejas, A.**, López-Velasco, G., Veladez, A.M., Sbodio, A., Artés-Hernández, F., Danyluk, M., Suslow, T.V. Establishment of critical operating standards for chlorine dioxide in disinfection of dump tank and flume water for fresh tomatoes. Submitted, May 2011.

- López-Velasco, G., **Tomás-Callejas, A.**, Sbodio, A., Artés-Hernández, F., Suslow, T.V. Chlorine dioxide dose, water quality and temperature affect the oxidative status of tomato processing water and its ability to inactivate *Salmonella*. Submitted, May 2011.

Other publications

- **Tomás-Callejas, A.**, Bariain, N., López, J.J., Robles, P.A., Artés, F. y Artés-Hernández, F. 2008. Radiación UV-C en brotes de hortalizas foliáceas: una alternativa a la desinfección con cloro en el procesado mínimo en fresco. *Alimentación, Equipos y Tecnología 231*, 46-49.

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- **Tomás-Callejas, A.**, Martínez-Hernández, G.B., Spooren, R., Artés F., Artés-Hernández, F. 2010. La desinfección con agua electrolizada preserva la calidad microbiológica, nutritiva y sensorial de brotes de Mizuna mínimamente procesados en fresco. *Revista Iberoamericana de Tecnología Postcosecha 11*, 204-213.