Estudio y modelización del efecto de procesos de descontaminación y desinfección sobre microorganismos patógenos en productos vegetales

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Tesis presentada por la Ingeniera Dña. Guiomar Denisse Posada Izquierdo para obtener el título de Doctora por la Universidad de Córdoba. Julio 2013

TITULO: *Estudio y modelización del efecto de procesos de descontaminación y desinfección sobre microorganismos patógenos en productos vegetales.*

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TÍTULO DE LA TESIS: "Estudio y Modelización del efecto de procesos de descontaminación y desinfección sobre Microorganismos patógenos en productos vegetales"

DOCTORANDO/A: Ing. Doña Guiomar Denisse Posada Izquierdo.

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La Doctorando ha realizado un programa formativo fundamentado en dos pilares básicos. El primero es el desarrollo de estancias a nivel nacional e internacional relacionadas con aspectos microbiológicos y de calidad sensorial del sector de productos IV, siendo de gran relevancia la estancia en centro CSIC-CEBAS (Murcia), Universidad de Gante (Bélgica) y Universidad de Michigan (EEUU). Todas ellas con importantes beneficios curriculares, de producción científica y formativa que han ayudado al desarrollo de la tesis en sus objetivos y tareas previstas. El segundo pilar es su dedicación al trabajo de laboratorio y en planta piloto, mostrando gran habilidad y conocimiento en el desarrollo de técnicas microbiológicas con patógenos tipo II, control de procesos tecnológicos en IV gama y análisis sensorial, todo ello con gran repercusión en la presente tesis. Por otro lado, dada la temática del trabajo de tesis, fundamentado en generar datos y aplicarlos en el desarrollo y propuesta de modelos predictivos, ha necesitado de una revisión, estudio y aplicación de conocimientos y conceptos matemáticos muy exhaustiva y rigurosa. Esto se ha traducido en una especialización del doctorando en tres ámbitos bien distintos, que son análisis microbiológico, tanto a escala de laboratorio como piloto y modelización matemática, garantizando de esta manera la calidad de su tesis y la continuidad del grupo de investigación en el ámbito de la microbiología predictiva.

Los trabajos presentados en la tesis han requerido un gran desarrollo logístico ya que muchos de ellos han sido desarrollados a una escala piloto. Dado la contribución de los centros de estancias, y a la necesidad de participación para llevar un proceso piloto, los artículos han incluido amplias autorías. Sin embargo, son todos ellos, de manera clara, demostración indiscutible del trabajo principal de la Doctorando, evidenciando en su desarrollo una gran capacidad de gestión y coordinación en planta, a la vez que desarrollaba el diseño experimental, proceso experimental de análisis y modelización de los datos.

Los trabajos han versado sobre el desarrollo de modelos de crecimiento e inactivación generados en productos vegetales y en medio artificial en condiciones comerciales y de laboratorio con gran aplicación en el sector industrial y gubernamental para el desarrollo de políticas de gestión de riesgos microbiológicas, como en el caso de *Escherichia coli* Verotoxigénico, estudiado en la presente tesis. A modo de compendio de trabajos científicos, la tesis presenta trabajos publicados o enviados a revistas del primer cuartil del ISI citation index, como podrían ser International of Food Microbiology, Food Microbiology, or Food Research International. No solo el documento de tesis tiene un valor científico de excelencia sino el bagaje y producción paralela que el doctorando ha generado con múltiples publicaciones en otros ámbitos de la microbiología predictiva como pueden ser en el sector cárnico y de evaluación de riesgos microbiológicos y que a su vez se han traducido en ponencias y participación en diversos y variados congresos nacionales e internacionales y en la colaboración en proyectos de investigación de carácter nacional (por ej. AGL 2008, Ministerio de Innovación y Ciencia) e internacional (por ej. Baseline VII European Program).

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 10 de Julio de 2013

Firma del/de los director/es

Fdo.: FERNANDO LÓPEZ

Fdo.: GONZALO ZURERA

A papi, mami, toño y mi Fer

El verdadero amor no es otra cosa que el deseo inevitable de ayudar al otro para que sea quien es

La realización de la Tesis Doctoral ha sido posible gracias a una beca Pre-Doctoral del Ministerio de Ciencia e Innovacion del Gobierno de España y del Proyecto de Excelencia AGL2008-03298 concedido al Grupo HIBRO de la Universidad de Córdoba.

Studying and modeling of the effect of decontamination and disinfection of pathogenic microorganisms in minimally processed vegetable products

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Título: Estudio y Modelización del efecto de procesos de descontaminación y desinfección sobre Microorganismos patógenos en productos vegetales

Año de Publicación: 2013

ISBN:

Tesis Doctoral mención Internacional dentro del programa de calidad del doctorado de la Universidad de Córdoba, Córdoba, España. Con los artículos científicos en inglés y resumen y conclusiones

Indice

Listado de abreviaturas y símbolos

Abreviaturas	Significado
ABS	Absorbancia
AEW	Acidic electrolysed water
ADN	Ácido desoxirribonucleico
AE	Agua electrolizada
AEM	Microorganismos Aerobios Mesófilos
AESAN	Agencia Española de Seguridad Alimentaria y Nutrición
AFHORLA	Asociación Española de Frutas y Hortalizas Lavadas, Listas para el consumo, nueva AFHORFES
AFHORFES	Asociación Española que agrupa a los fabricantes de Frutas y Hortalizas Lavadas Listas para su empleo, IV Gama, integrada en FEPEX. AFHORFES (antigua AFHORLA)
AHAs	Ácidos haloacéticos
AEW	Agua Electrolizada Alcalina
AINIA	Centro Tecnologico de la Industria Agroalimentaria, Valencia
ANICE	Asociación Nacional de Industrias de la Carne de España
ANOVA	Análisis de la varianza
Anova	Analisis de varianza
APHA	American Public Health Association (Asociación Americana de salud pública)
APPCC	Análisis de Peligros y Puntos de Control Críticos
ARM	Análisis del Riesgo Microbiológico
ATCC	American Type Culture Collection (Colección Americana de Cultivos Tipo)
atm	Atmosphere
ATSDR	Agency for Toxic Substances and Disease Registry
aw	Activity water (Actividad de agua)
BAL	Bacterias ácido-lácticas
BHI	Brain Heart Infusion (Caldo Infusión Cerebro Corazón)
BOE	Boletín Oficial del Estado
BPA	Buenas Prácticas agrícolas
BPH	Buenas prácticas de higiene
BPM	Buenas prácticas de manufactura
C/NC	Probabilidad de Crecimiento/No Crecimeinto

$Ca(ClO)_2$	Hipoclorito de Calcio
Ca^{2+}	Ión calcio
CCA	Comisión del Codex Alimentarius
CCFRA	Campden and Chorleywood Food Research Association
CDC	Centers for Disease Control and Prevention (Centros para el control y la Prevención de Enfermedades, Estados Unidos)
CEBAS	Centro de Edafología y Biología Aplicada del Segura
CECT	Colección Española de Cultivos Tipo
CF	Coliformes fecales
CFSAN	Center for Food Safety and Applied Nutrition (Centro de Seguridad Alimentaria y Nutrición Aplicada; Estados Unidos)
cfu	Colony forming units (Unidades formadoras de colonias)
CH_3CO_2H	Ácido Acético
CH_3CO_3H	Ácido peroxiacético
Cl^-	Ión cloruro
Cl_2	Cloro molecular
ClO^-	Ión hipoclorito
ClO_2	Dióxido de Cloro
ClO_2^-	Ión clorito
ClO_3^-	Ión clorato
cm	Centímetro
cm^2	Centímetro cuadrado
CO_2	Dióxido de Carbono
COT	Comittee on Toxicity
CSA	Clorito de sodio acidificado
CSIC	Consejo Superior de Investigaciones Científicas
CT	Coliformes totales
DISAL	Diseño en el sector alimentario, del Centro Tecnológico AINIA
DQO	Demanda Química de Oxígeno
E. coli	*Escherichia coli*
ECDC	European Centre for Disease Prevention and Control (Centro Europeo para la Prevención y Control de Enfermedades)
ECEH	*Escherichia coli* Enterohemorrágica
ECRM	Evaluación Cuantitativa de Riesgo Microbiológico

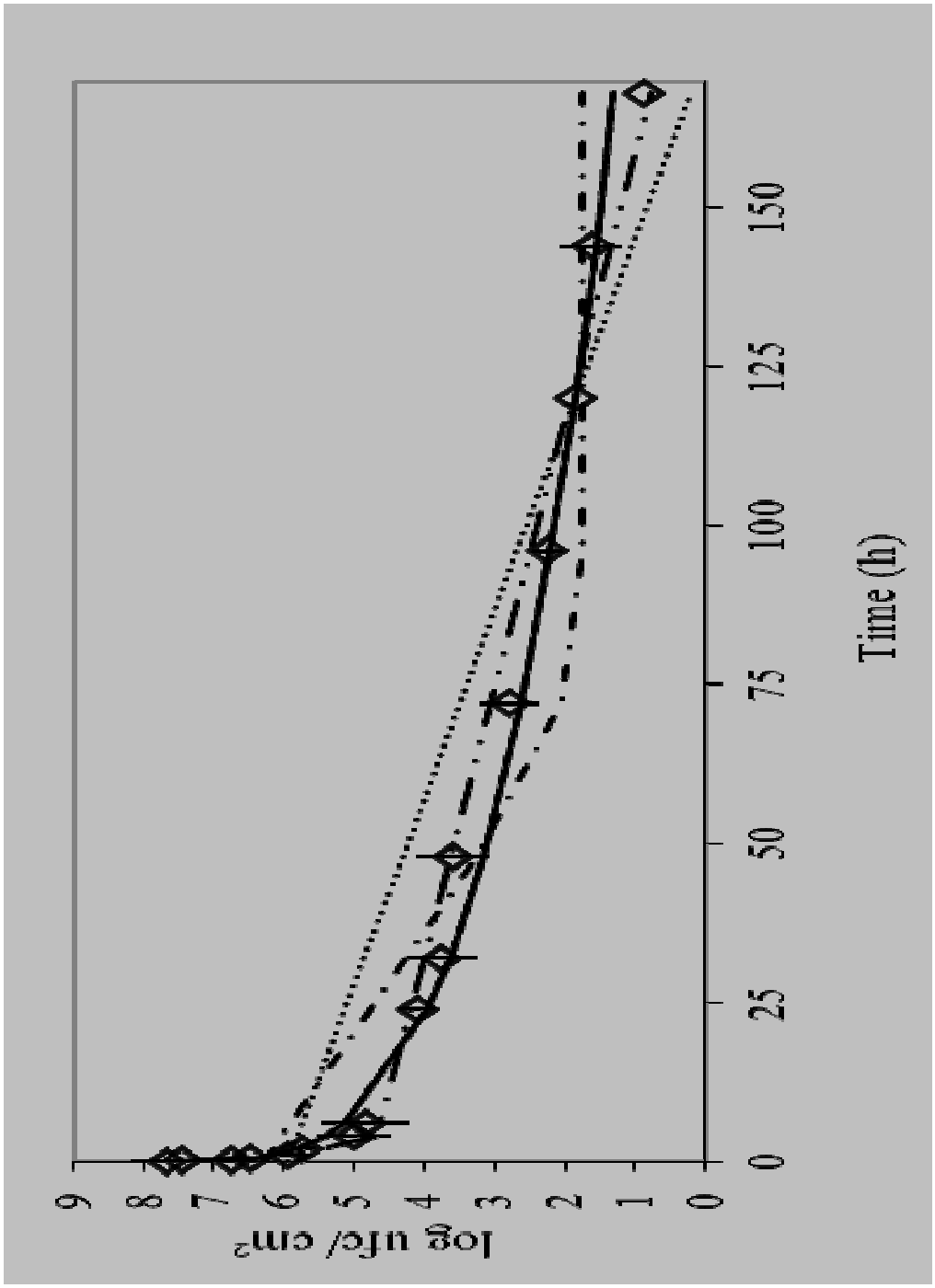
ECVT	*Escherichia coli* Verotoxigénica
EEUU	Estados Unidos
EFFAT	European Federation of trade unions in the Food, Agriculture and Tourism (Federación Europea de Sindicatos de Alimentos, Agricultura y Turismo)
EFSA	European Food Safety Authority (Agencia Europea de Seguridad Alimentaria)
EMAP	Equilibrium modified atmosphere packaging
FAO	Food and Agriculture Organisation of the United Nations (Organización de las Naciones Unidas para la Alimentación y la Agricultura)
FDA	Food and Drug Administration (Agencia de Alimentos y Medicamentos;Estados Unidos)
FEPEX	Federación Española de Asociaciones de Productores y Exportadores de Frutas, Hortalizas, Flores y Plantas
FN	Falsos negativos (%) (Indice de bondad de ajuste de modelo de Probabilidad de crecimiento)
FP	Falsos positivos (%) (Indice de bondad de ajuste de modelo de Probabilidad de crecimiento)
FSA	Food Standart Agency (Agencia de Estándares de Alimentos; Reino Unido)
g	Gramo
h	Hora
H^+	Ión hidrógeno
H_2O	Agua
H_2O_2	Peróxido de hidrógeno
HANs	Haloacetonitrilos
HCl	Ácido clorhídrico
HClO	Ácido hipocloroso
$HClO_2$	Ácido cloroso
HOSCN	Ácido hipotiocianoso
HPA	Health Protection Agency (Agencia de Protección para la Salud)
Hz	Hercio, pulses per second
IARC	International Agency for Research on Cancer (agencia internacional del studio del cancer)
ICMSF	International Comission on Microbiological Specification in

	Foods (Comisión Internacional de Especificaciones Microbiológicas en Alimentos)
IFPA	International Fresh-cut Produce Association (asociacion internacional de productos frescos cortados)
IFR	Institute of Food Research, UK (Instituto de Investigación de Alimentos, Reino Unido)
IFT	Institute of Food Technologists (institute de tecnologia de los alimentos)
IL	Inoculum level (nivel de inóculo, log ufc/ml)
INE	Instituto Nacional de Estadística
ISO	International Organisation for Standardization (Organización Internacional para la Estandarización)
kg	Kilogramo
L	Litro
LAB	Lactic acid bacteria (Bacterias Acido Lacticas)
lag o λ	Tiempo de adaptación microbiana o de latencia (h)
LMG	Laboratorium Microbiologie, Universiteit Gent, Belgium
log	log10
logdiff	Diferencia logarítmica
m	Valor umbral del número de bacterias. El resultado se considerará satisfactorio si todas las unidades que componen la muestra tienen un número de bacterias igual o menor que m.
M	Valor límite del número de bacterias. El resultado se considerará no satisfactorio si una o varias unidades que componen la muestra tienen un número de bacterias igual o mayor que M.
MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
MAP	Modified atmosphere packaging(Envasado en atmosfera modificada)
mg	Miligramo
min	Minuto
ml	Mililitro
MP	Microbiología Predictiva
MRS	De Man, Rogosa y Sharpe
MSE	Mean Square Error (Error Cuadrático Medio)
n	Número de muestras/réplicas

N	Numero de microorganismos después del tratamiento
N_0	Concentración de inóculo inicial
N.A.	Datos no adecuados para el ajuste
Na^+	Ión sodio
NaCl	Cloruro sódico
NaClO	Hipoclorito de sodio
$NaClO_2$	Clorito de sodio
ND	No hay datos
NEW	Neutral electrolysed oxidizing water
nm	Nanómetro
No	Number of microorganisms before treatment
O_2	Oxígeno
O_3	Ozono
OCM	Organización Común de Mercados Agrícolas
OD	Optical Density (Densidad Óptica)
OH^-	Ión hidroxilo
OMS	Organización Mundial de la Salud
$OSCN^-$	Ión hipotiocianito
OVQ	Overall visual quality
P	Probabilidad de crecimiento
p	Probabilidad de significación estadística
PAL	Fenilalanina-amonio-liasa
PCA	Plate Count Agar (Agar de recuento en placa)
PCC	Punto Crítico de Control
PCR	Reacción en cadena de la polimerasa
PERSEO	Programa piloto Escolar de Referencia para la Salud y el Ejercicio, contra la Obesidad
pH	Potencial de Hidrógeno
P3ARRT	Herramienta para calificar el riesgo Patógeno-Producto
POA	Procesos de oxidación avanzados
POD	Peroxidasa
ppm	Partes por millón (mg/L)
PPO	Polifenol-oxidasa
PSS	Physiological saline solution (solusion salina)
r	Coeficiente de correlación de Pearson
R^2	Coeficiente de determinación

HR	Porcentaje de humedad relativa
RTE	Listo para el consumo (VI Gama)
s	Segundos
S.D.	Standard deviation (desviación estantar)
SEA	Staphylococcal enterotoxin A (enterotoxina staphylococica)
T	Temperatura (ºC)
t	tiempo (h)
TBX	Tryptone Bile-X Glucoronide (Agar Bilis Triptona)
td	Tiempo de detección (h)
Teff	Temperatura Estática Efectiva (ºC)
THMs	Trihalometanos
*t*inc	Tiempo de incremento (h)
TiO_2	Dióxido de titanio
TSB	Tryptone Soja Broth (Caldo de Triptona Soya)
t-test	Student t-test(test- estadístico)
UE	Unión Europea
ufc	Unidades formadoras de colonias
ufc/g ó ml	Unidades formadoras de colonias/gramo ó mililitro
UK	United Kingdom
USA	United States of America
USDA	United States Department of Agriculture (Departamento de Agricultura de Estados Unidos)
USDA-ARS	United States Department of Agriculture-Agricultural Research Service Departamento de Agricultura de Estados Unidos-Servicio de InvestigaciónAgrícola
UV	Luz ultravioleta
UV-C	Part of the electromagnetic spectrum with wavelengths in the range 200-280 nm
v/v	Volume/volume
Volts	Voltage
VTEC	Verotoxigenic Escherichia coli (Escherichia coli verocitoxigénico)
W	Watts
w/v	Weight/volumen (peso/volumén)
λabs	Tiempo de latencia, h (Parámetro de crecimiento de Absorbancia)
µg	Microgram

µabs	Tasa de absorbancia, h-1(Parámetro de crecimiento de absorbancia)
%	Porcentaje
µg	Microgramo
µl	microlitro
µm	micrometro
µmax	Tasa máxima de crecimiento, h-1
ºC	Grado Celsius
A	Amperes


log ufc/ cm²
0
1
2
3
4
5
6
7
8
9
0
25
50
75
100
125
150
Time (h)

Abstract/Resumen

The present thesis is aimed at quantifying the effect of different food process on fate of enteric pathogenic bacteria, *Escherichia coli* O157:H7 and *Salmonella* spp. in vegetables (*Introduction*). The main contribution of this thesis lies in providing quantitative tools based on predictive models to support microbial risk management systems in the Vegetable Industry. In first instance, a mathematical model describing cross contamination of *E. coli* O157:H7 during processing of fresh-cut vegetable was developed and simulated *in silico (Chapter I)*. Three different scenarios, named S1, S2, and S3, were considered to represent the initial concentration on the contaminated batch entering the processing line which corresponded to 0.01, 1 and 100 cfu/g, respectively. Given the low initial levels, differences between scenarios were only observed in prevalence and not in concentration ($p< 0.001$). The model evidenced that cross contamination was possible in all simulated scenarios. Given the importance of quantifying the effect of new disinfection treatments as means of avoiding cross contamination during washing step, the efficacy of an electrochemical treatment in water disinfection, using boron-doped diamond electrodes, was studied together with its suitability for the fresh-cut produce industry *(Chapter II)*. Tap water (TW), and TW supplemented with NaCl (NaClW) containing different levels of organic matter around 60, 300, 550±50 and 750±50 mg/L; combined and total chlorine, pH, oxidation-reduction potential, COD and temperature were monitored during the treatments and obtained in this optimum conditions reductions of 5 log units of *E. coli* O157:H7. Results provided suitable base to develop predictive models describing reduction as a function of time at the different studied conditions, the Weibull model obtaining a good performance. The effect of disinfection treatment on subsequent growth of *E. coli* O157:H7 in fresh-cut leafy vegetables was studied considering traditional and alternative treatments, based on chlorine and electrolyzed water, respectively *(Chapter III and IV)*. In the first case, fresh-cut iceberg lettuce inoculated with *E. coli* O157:H7 was submitted to chlorine washing (150 mg/mL) and modified atmosphere packaging on laboratory/pilot scale. Potential growth of the pathogen was

assessed at 4, 8, 13 and 16 °C with multiple replicates. The pathogen was able to grow at temperatures ≥8 °C, although at lower temperatures, growth data presented a high variability between replicates. For neutral electrolyzed water treatment, the experimental set-up was the same as that used in study dealing with chlorine. Results indicated that growth in lettuce treated with EW was lower than that observed when treated with chlorinated water. In both experiments, a Ratkowsky-type model was proposed to account for the relationship between temperature and growth rate. The survival capacity of *E. coli* O157:H7 and *Salmonella* spp. were assessed in stainless steel soiled with different sterile vegetable juices simulating soiling conditions in the IV gamma produce factories *(Chapter V).* Results indicated that both pathogens could be recovered until 192 and 168 h, respectively from surfaces soiled with chard, red cabbage, iceberg lettuce and romaine lettuce. However, in spinach and parsley juice substrates, microorganisms were not detected after 48 h. This survival time was much lower than that obtained in saline solution (120 h) used to simulate cleaning conditions, suggesting the presence of antimicrobial substances. Furthermore, based on generated data, survival models were proposed to simulate survival on equipment surface. In general, the Weibull model and the biphasic models were the ones with the best performance. These models could be used in risk assessment studies to represent cross contamination scenarios simulating the number of viable cells on surfaces available to be transferred to vegetables. Finally, an attempt to simulate growth of *E. coli* O157:H7 in broth was made based on the use of sterile vegetable juices (iceberg lettuce, chard, spinach, parsley and romaine lettuce) *(Chapter VI).* This study is based on the hypothesis that pathogenic bacteria could be often found in injuries of vegetable tissue, thereby they would be exposed to vegetable cell content. The experiments were carried out in Bioscreen C equipment and growth rates were estimated on inoculated vegetable juices based on absorbance measurements over time. Finally, secondary models describing the dependence between temperature and kinetic parameters were derived. The results indicated the capacity of growth depended on type of vegetable juice and temperature. Moreover, some vegetable juices did not support growth suggesting, in these cases, the presence of inhibitory substances such as a high content in polyphenolic compounds.

La presente tesis tiene como objetivo principal cuantificar el efecto de diferentes procesos alimentarios sobre el comportamiento de las bacterias patógenas, *Escherichia coli* O157: H7 y *Salmonella* spp., en vegetales (*Introducción*). Por tanto, la principal contribución de esta tesis consiste en proporcionar herramientas cuantitativas basadas en modelos predictivos como apoyo a los sistemas de gestión de riesgos microbiológicos en la industria de vegetales. En primera lugar, se desarrolló un modelo matemático de contaminación cruzada de *E. coli* O157:H7 durante el procesamiento de vegetales IV gama que fue simulado en *in-silico* (*Capítulo I*). Tres escenarios diferentes fueron contemplados, denominados S1, S2, y S3, y que correspondieron a diferentes concentraciones iniciales en un lote contaminado a la entrada en la línea de procesos, esto es 0,01, 1 y 100 ufc / g, respectivamente. Dado los bajos niveles simulados, las diferencias entre escenarios sólo se observaron para los valores de prevalencia resultantes, y no para concentración ($p < 0,001$). En resumen, el modelo evidenció que la contaminación cruzada fue posible en todos los escenarios probados. Dada la importancia de cuantificar el efecto de los nuevos tratamientos de desinfección utilizados para minimizar la contaminación cruzada durante la etapa de lavado, la eficacia de un tratamiento electroquímico en la desinfección del agua, usando electrodos con boro de diamante, fue estudiado junto con su idoneidad para su aplicación en la industria de productos de IV Gama (*Capítulo II*). Se estudio el agua del grifo (TW) y TW suplementada con NaCl (NaClW), ambas con diferentes niveles de materia orgánica (60, 300, 550 ± 50 y 750 ± 50 mg / L). El cloro libre y total, pH, potencial oxidación-reducción, COD y la temperatura fueron analizados durante los tratamientos. En condiciones óptimas se obtuvieron reducciones de 5 unidades logarítmicas de *E. coli* O157: H7. Los resultados proporcionan la base adecuada para desarrollar modelos predictivos que describen la reducción en función del tiempo a los diferentes niveles de materia organica. El modelo de Weibull fue el modelo que obtuvo el mejor ajuste a los datos de supervivencia. El efecto de los tratamientos de desinfección sobre el crecimiento posterior de *E. coli* O157: H7 en vegetales IV gama también fue estudiado considerando tratamientos tradicionales y alternativos, como el uso de cloro y agua electrolizada, respectivamente (*Capítulo III y IV*). En el primer caso, lechuga iceberg IV gama inoculada con *E. coli* O157: H7 fue sometida a lavado con agua clorada (150 mg / ml) y envasada en atmósfera modificada en un ensayo llevado acabo a escala de laboratorio/piloto. El potencial de crecimiento del patógeno fue evaluado a 4, 8, 13 y 16 °C con múltiples repeticiones. El patógeno fue capaz de crecer a temperaturas ≥ 8 °C, aunque a temperaturas bajas, los datos presentaron una gran variabilidad entre repeticiones. Para el tratamiento de agua electrolizada neutra (AEN), el diseño experimental fue el mismo al utilizado en el estudio de tratamiento con cloro. Los resultados indicaron que el crecimiento del patógeno en la lechuga tratada con AEN fue menor que la observada con agua clorada. En ambos experimentos, se propuso un modelo tipo Ratkowsky para representar la relación entre la temperatura y la tasa de crecimiento. La capacidad de supervivencia de *E. coli* O157: H7 y

Salmonella spp., fue evaluada en acero inoxidable impregnado con diferentes estractos estériles de vegetales, simulando condiciones de suciedad en las industrias de IV gama (*capítulo V*). Los resultados indicaron que ambos patógenos podría ser recuperados hasta 192 y 168 h después de inocularlos en las superficies con extracto de acelga, col lombarda, lechuga iceberg y lechuga romana. Sin embargo, en las superficies con los extractos de espinacas y perejil no se detectaron microorganismos después de 48 h. Este tiempo de supervivencia, fue mucho menor que el obtenido en solución salina (120 h), utilizada para simular las condiciones de limpieza, lo que sugeriría la presencia de sustancias antimicrobianas en los extractos de espinaca y perejil. Además, basados en los datos generados, se propusieron modelos de supervivencia para simular la capacidad de supervivencia en superficies de acero. En general, el modelo de Weibull y el modelo bifásico fueron los que representaron mejor los datos observados. Estos modelos podrían utilizarse en estudios cuantitativos de evaluación de riesgos y en el análisis de escenarios de contaminación cruzada, ya que permiten estimar el número de células viables en las superficies que se encuentran disponibles para ser transferidas a los vegetales. Por último se simuló el crecimiento de *E. coli* O157: H7 en caldo utilizando extractos estériles de vegetales (lechuga, acelga, espinaca, perejil y la lechuga romana) (*Capítulo VI*). Este estudio se basa en la hipótesis de que las bacterias patógenas podrían encontrarse en lesiones de tejidos vegetales, con lo que estarían expuestas al contenido celular del vegetal. Los experimentos se llevaron a cabo en un equipo Bioscreen C a partir de los extractos vegetales inoculados. Las tasas de crecimiento se estimaron a través de la medicion de la absorbancia a lo largo del tiempo. Finalmente, los modelos secundarios propuestos describieron la relación matemática entre la temperatura y parámetros cinéticos. Los resultados indicaron que la capacidad de crecimiento fue dependiente del tipo de extracto vegetal y de la temperatura. Por otra parte, algunos extractos vegetales no permitieron crecimiento, lo que sugiere, en estos casos, la presencia de sustancias inhibidoras como podría ser un alto contenido en compuestos polifenólicos.

Introducción

Vegetales de IV Gama

Definición de IV Gama

Los vegetales IV gama son productos vegetales, frutas y hortalizas "frescos", que han podido ser objeto de troceado, corte o cualquier otra operación relativa a la integridad física del producto, (como por ejemplo: preparados, lavados y envasados). Debido a que los producto IV Gama se encuentran listos para consumirse, AFHORFES destaca que estos productos tienen las peculiaridades de mantener sus propiedades naturales y frescas, sin incorporar ningún tipo de aditivo ni conservante, encontrándose durante toda la cadena de producción bajo condiciones de refrigeración. El término IV Gama está relacionado con el nivel tecnológico empleado. La I Gama corresponde a las frutas y hortalizas frescas tradicionales; la II Gama corresponde a las hortalizas en conserva; la III Gama son las hortalizas congeladas y la V Gama son hortalizas cocidas y conservadas. También los productos IV Gama se les conoce comercialmente como alimentos mínimamente procesados (Artés y col., 2009).

Orígenes de la IV Gama

La idea de IV Gama surgió en los Estados Unidos en los años 70, originada para satisfacer las necesidades de los restaurantes de comida rápida y de los establecimientos de venta de ensaladas. En principio, la materia prima más utilizada fue la lechuga iceberg, con un tiempo de conservación no superior a los 4 días. Una década más tarde los productos IV Gama se abren camino en los país Europeos, siendo los pioneros: Suiza, Holanda y Bélgica principalmente en fábricas familiares de agricultores que proporcionan un valor agregado a su propia producción; realizando muchas de las operaciones manualmente. Por otro lado, y con una tendencia más industrial Francia se une a la producción de las frutas y hortalizas IV Gama, mientras que Alemania y los Países escandinavos, aplicando una organización simple y enfocando la producción de IV Gama para grandes colectivos. Posteriormente la producción de vegetales de IV Gama se extendió a otros países, como Italia, Gran Bretaña y finalmente España, donde Navarra fue la comunidad pionera en comercializar este tipo de producto, hasta extenderse a otras comunidades hortofrutícolas como Murcia, Comunidad Valenciana, Andalucía y Cataluña (Carbonell, 1990, Sánchez Pineda, M. 2003).

Esquema general productivo

Las principales fases en el proceso de obtención de vegetales de IV Gama se representan en Figura 1. Según Gil y Gorny, (2003), e**l proceso de fabricación** de frutas y hortalizas de IV Gama comienza en el campo, donde se cultiva la materia prima en las mejores condiciones (de seguridad alimentaria, calidad y respeto al medio ambiente). La **recolección** del material vegetal se hace en las óptimas condiciones higiénicas, con el color y textura adecuados, y en su grado justo de madurez. Desde el punto de vista industrial, el proceso comienza con la **recepción y almacenamiento** de frutas y

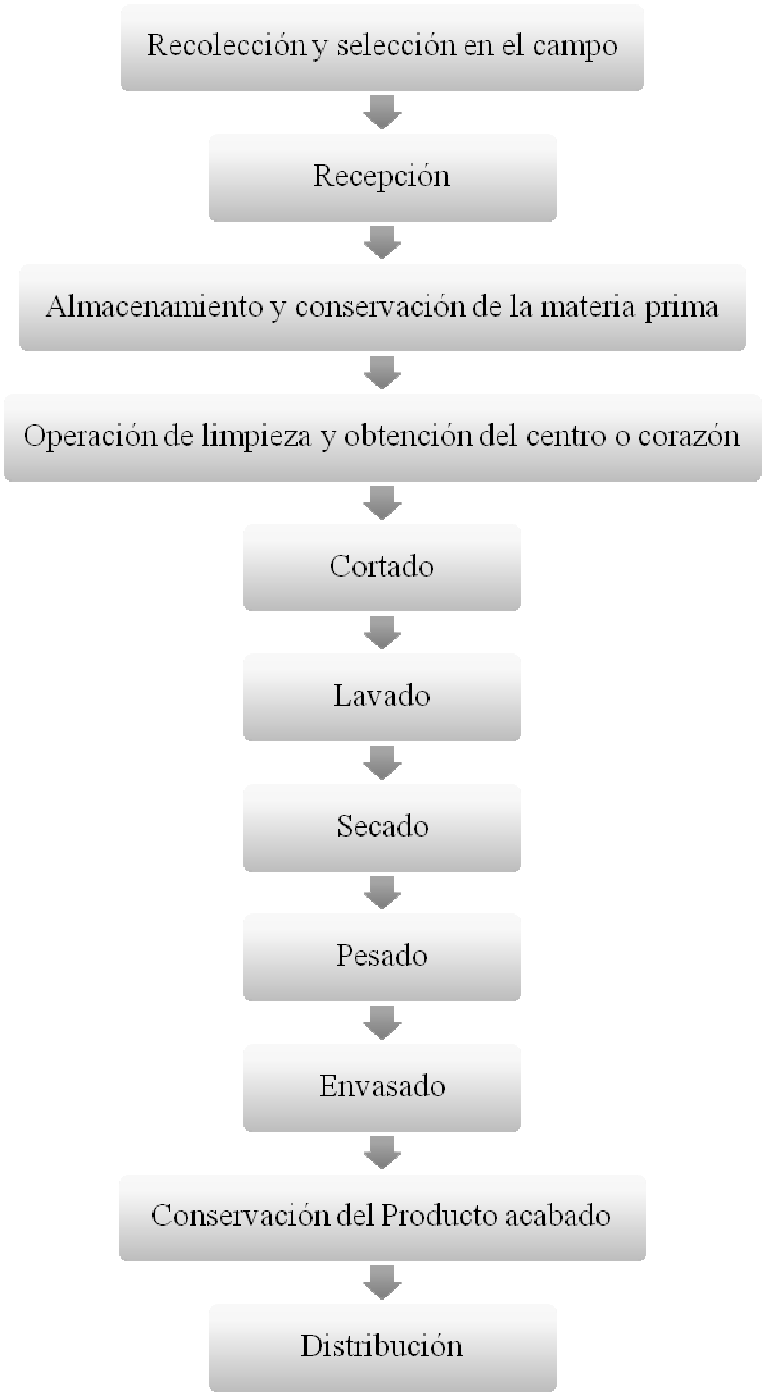


Figura 1: Diagrama de flujo general de una planta de procesado de vegetales de hoja IV Gama (Adaptado de Gil y Gorny, 2003).

hortalizas en las fábricas. La **limpieza,** que no es más, que la selección de la parte óptima (puede suponer una pérdida del 20 al 70% del producto), operación que se realiza de manera manual. El **cortado** consiste en trocear el material vegetal hasta el tamaño comercial del producto. La fase de **lavado** se realiza en dos fases intensivas, con el fin de eliminar la suciedad del campo. El **secado** superficial es fundamental para la conservación del producto y se efectúa mediante la eliminación del exceso de agua que habitualmente se realiza a través del uso de centrifugas industriales. El **pesado y envasado** de los productos troceados es la fase final del proceso; (en función del producto, se busca el envase más adecuado, que incluye desde bolsas a barquetas,

tarrinas o bandejas; siempre son envases transparentes para que el consumidor pueda percibir la frescura y calidad del producto). El **almacenamiento** se realiza en condiciones de refrigeración hasta su consumo. La temperatura recomendada en todo el proceso, desde que se recolecta la materia prima hasta la colocación en el punto de venta debe oscilar entre 1 y 4 ºC. Los productos de IV Gama deben encontrarse refrigerados para conservarlos en sus óptimas condiciones, hasta el momento del consumo (AFHORFES, 2013). De las materias primas más utilizadas en la elaboración de productos IV Gama destacan las lechugas, para ensaladas, con producciones importantes a nivel mundial.

Tendencias

En los últimos años el grado de aceptación y demanda de los vegetales IV Gama, ha ido incrementándose progresivamente, hasta el punto de ser alimentos frecuentes en las compras básicas; ya que como es más que conocido: "el consumo de frutas y hortalizas forma parte de los elementos claves en la dieta mediterránea" (OMS y FAO, 2003). En este sentido, las recomendaciones de la OMS y FAO (2003), sitúa el consumo mínimo aconsejable de frutas y vegetales entorno a los 400g/día, así como también el Ministerio Agricultura, Alimentación y Medio Ambiente Español, a través de la promoción de la campaña de: "5 al día", (de la asociación para el consumo de frutas y hortalizas frescas) promueven al consumo de estos productos, en la búsqueda de un estilo de vida más sano, (MAGRAMA, 2007)

Sector

El sector de vegetales de IV Gama es uno de los mercados más prometedores de la alimentación como consecuencia de diferentes factores: sus beneficios nutricionales, a la carencia de tiempo para preparación, y a la necesidad de hacer las comidas fuera del hogar siendo el 33 % de la alimentación de los españoles fue Extradoméstica en el año 2012, (MAGRAMA, 2012). Según los datos aportados por FEPEX, el sector de la IV Gama supone un volumen de negocio en torno a los 180-200 millones de euros. En el 2010, en productos IV Gama en España se comercializaron 70,6 mil toneladas entre frutas y hortalizas listas para su consumo, de las cuales, 69,1 mil toneladas fueron de vegetales IV Gama (según AFHORFES integrada en FEPEX). La producción agrícola destinada como materia prima para vegetales IV Gama ocupa el 10% de la zona hortofrutícola española y se encuentra repartida en los siguientes productos: 60% lechugas (varias), 17% Ensaladas (mezclas de varias hortalizas), 7% espinaca, 3% brotes, 3% zanahoria, y el restante porcentaje en otros productos IV Gama (AINIA, 2007).

Producción y Comercio del Sector

Según AFHORFES, la producción nacional de vegetales IV Gama ha ido en aumento de forma constante en los últimos años, impulsada principalmente por: 1) la creciente innovación del sector, 2) la mejora en la integración de todos los componentes de la cadena productiva, 3) incremento en el rendimiento productivo, 4) un aumento en la calidad del producto, 5) así como también un incremento en su vida útil (Anónimo, 2013). El balance comercial se ha visto afectado por la crisis económica mundial. Y por la eficiencia del sector, que origina más oferta que demanda en España. Por ello, los datos que se conocen sitúan en el 2012, una caída con respecto al 2011 del 76,47 % en las importaciones y en las exportaciones la disminución ha sido de 20,17% (siendo principalmente en Frutas IV Gama) (MERCASA, 2012). Cabe destacar que el comercio

nacional se incrementó 1,65% en el 2012, con una alza de 2,51% en la distribución y una baja de 2,11% en el canal HORECA, (Anónimo, 2013).

Consumo

En el 2011, el consumo medio de frutas y hortalizas de IV Gama fue del 4% del total de hortalizas y verduras consumidas, que implica 3.35 kg per cápita (MAGRAMA, 2012) (Figura 2). En cambio, los datos preliminares del 2012 situan el consumo de vegetales IV Gama en 1,44% por debajo del consumo del 2011, consumiéndose un total per capita 3,30 kg/mes (Anónimo, 2013). El consumo per capita en España está muy por debajo del consumo medio per capita en EE.UU (30 kg/año) o Francia (6 kg/año). Además, el consumo medio de vegetales IV Gama respecto a vegetales fresco corresponde en UE al 7% y en EE.UU., al 85% (AINI, 2010).

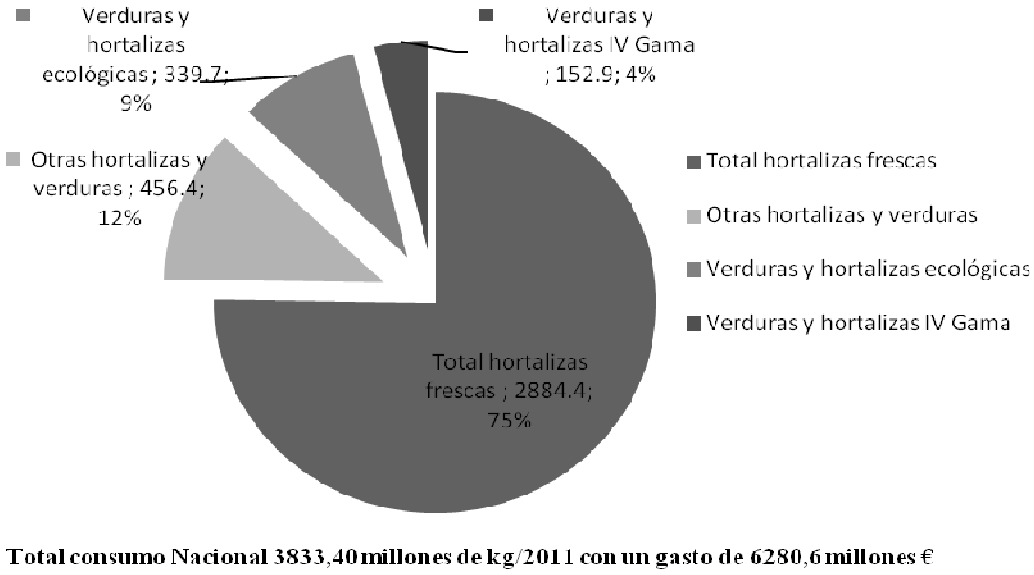


Figura 2: Porcentaje del consumo en los hogares Españoles en el año 2011. (MAGRAMA, 2012).

Evolución Sector

Es interesante evaluar la evolución del sector en los últimos 5 años, para ello, en la Figura 3 se representa el comportamiento de los datos de consumo a lo largo del periodo 2007-2011. El año 2007 se sitúa como punto de partida con un valor de 100 para representar el consumo de vegetales tanto frescos como procesados. Respecto a este volumen inicial, en vegetales IV Gama, el consumo aumentó en 2 puntos en el 2008, incrementandose levemente en el 2009, para posteriormente mostrar una tendencia a la baja en los años siguientes. En cambio, en vegetales frescos de hoja, el consumo se incremento en el 2008, superando a los vegetales IV Gama en 4 puntos para en el siguente año (2009) disminuir igualándose a estos productos. Por otro lado, la comercialización del sector experimentó en el 2010, una subida del 6% en vegetales IV Gama respecto al año 2009. El sector de la distribución en el 2010 demandó el 81% de la producción total de vegetales IV Gama, dirigiendo un 19% al sector de la red HORECA, e incrementandose este en un 3,5% respecto al año anterior (FEPEX, 2013; AINIA, 2011; Anónimo, 2013).

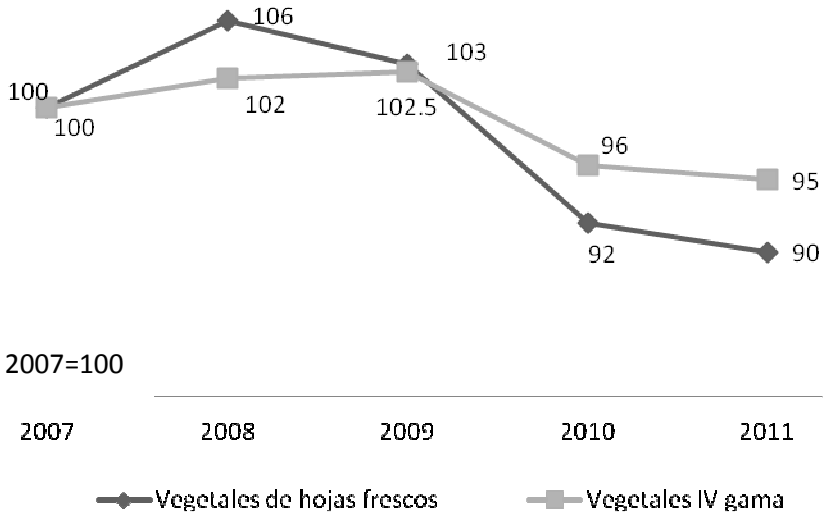


Figura 3**:** Evolución de consumo de vegetales en España 2009-2011 (MERCASA, 2012)

Seguridad Microbiológica de los Vegetales IV Gama

Fundamentos

Los alimentos pueden ser portadores de enfermedades tanto en su estado fresco o crudo como una vez procesados. Los peligros transmitidos por los alimentos pueden ser: físicos, químicos y biológicos (FAO, 2003). Los peligros biológicos en vegetales los constituyen bacterias, hongos, virus y otros parásitos microscópicos (Doyle y Erickson, 2008; FAO/OMS, 2008a; Harris y col., 2003; OMS, 1998; Sivapalasingam y col., 2004). A este respecto existe la percepción actual que los casos de toxiinfecciones asociados con el consumo de frutas y hortalizas ha aumentado en los últimos años (Doyle y Erickson, 2008). Aunque esta percepción puede deberse a otros factores y no en si a un incremento del número de casos se indica como una posible explicación para este fenómeno (Doyle y Erickson, 2008; FAO, 2008c; EFSA, 2007b):

- Mayores controles de vigilancia de los productos frescos y de las enfermedades transmitidas por los alimentos.
- Incremento de casos por la ampliación del comercio mundial gracias a la globalización.
- A las continuas mejoras tecnológicas en el sector productivo que aumentan la vida útil de los alimentos procesados.
- Aumento de la demanda de alimentos y la aparición de estilos de vida dinámicos que requieren alimentos listos para ser consumidos.

En contraposición a la afirmación anterior, hay estudios que indican una escasa presencia de microorganismos patógenos en frutas y hortalizas frescas en diferentes puntos de la cadena alimentaria (Johannessen y col., 2002; FDA, 2003; EFSA, 2006; Johnston y col., 2006; Arthur y col., 2007; EFSA, 2007a). A pesar de estos datos, las autoridades han recomendado que no se puede dejar de controlar la presencia bacteriana, puesto que, hasta la actualidad no existe un proceso para vegetales IV Gama capaz de eliminar completamente la carga microbiana (Abadias y col., 2008a; EFSA 2009)

Requerimientos de la seguridad microbiana en vegetales IV Gama

La seguridad microbiológica de los vegetales IV Gama radica en la calidad de la materia prima, en la eficiencia de la desinfección y control de la contaminación cruzada durante toda la cadena productiva. Por tanto, es necesario la implantación de programas de higienización que permitan reducir la flora natural procedentes de la producción agrícola. La actividad microbiana se puede mantener bajo control gracias a los procesos de higienización (mecánico o químico o antimicrobianos naturales) aplicadas de manera estricta durante las etapas de producción y una adecuada conservación en atmósfera modificada en condiciones de refrigeración durante el almacenamiento (Gil y Gomy, 2003). Para cumplir con estas condiciones de salubridad; se debe dar inicio con el cumplimiento de los requisitos relativos a las Buenas Prácticas Agrícolas (BPA), Buenas Prácticas de Fabricación (BPF) y Buenas Prácticas de Distribución (BPD); ya que todos estos procedimiento tienen como objetivo minimizar el riesgo de contaminación de la materia prima y los productos de IV Gama y a su vez garantizar su calidad. En este sentido, la Comisión del Codex Alimentarius publicó un código de Buenas Prácticas de Higiene (BPH) para las frutas y hortalizas frescas (CAC, 2003).

Brotes de toxiinfecciones alimentarias asociados a vegetales

El último informe de EFSA (2012) refleja un total de 5262 brotes de origen alimentario en la UE para el año 2010, con 43473 casos humanos, 4695 hospitalizaciones; 25 muertes; siendo estos similares a los reportados en el 2009. Este informe resalta que *Salmonella* es el microorganismo con mayor incidencia, en torno al 30.5% de todos los casos en vegetales y que han ido incrementándose a lo largo de los últimos años (EFSA, 2012). Los vegetales de hojas se han visto involucrados en números brotes durante los últimos años. Un resumen de los datos más importantes sobre brotes asociadas a vegetales ha sido recogido en la Tabla 1. Basada en esta tabla y en los datos aportados por EFSA (2012), los patógenos más frecuentes asociados a toxiinfecciones por vegetales fueron *Salmonella* y *Escherichia coli* verotoxigénico. Por ello, la preocupación sobre la seguridad alimentaria de estos productos toma cada vez mayor relevancia. Así, la FAO/OMS (2008 a,b,c) realizó un informe sobre los riesgos microbiológicos en frutas, hortalizas, especias y hierbas medicinales, incluyendo en dicho informe guías sobre las opciones de mitigación.

Tabla 1: Brotes de patógenos en vegetales a nivel mundial en los últimos años**.**

Año	Microorganismo	Alimento	nº de Casos	Lugar	Referencia
2012	*Escherichia coli* O157:H7	Lechuga romana	58	EE.UU	Centro de Control y Prevención de enfermedades (CDC,2012)
2011	*Escherichia coli* O104:H4	Brotes de semillas germinadas	3910	Alemania y 12 países europeos	Boletín Semanal Epidemiológico (2011)
2011	*Escherichia coli* O157:H7	Lechuga romana	60	EE.UU	CDC (2011)
2011	*Shigella sonnei*	albahaca fresca	46	Noruega	Guzman-Herrador (2011)
2010	*Escherichia coli*(enterotoxigénica) y *Norovirus*	Lechuga	260	Dinamarca	Ethelberg y col. (2010)
2010	*Escherichia coli* O145	Lechuga	12	EE.UU	Schreck (2010)
2007	*Escherichia coli* O157	Lechuga IV Gama	50	Holanda , Islandia	Friesema y col (2008)
2007	*Salmonella*	Brotes de alfalfa	51	Suecia	Werner y col (2007)
2006	*Escherichia coli* O157:H6	Ensalada IV Gama	150	EE.UU.	FDA(2006)
2006	*Escherichia coli* O157:H7	Espinaca baby	205	EE.UU., Canada	Jay y col. (2007)
2006	*Escherichia coli* O145	Lechuga romana IV Gama	26	EE.UU	CDC (2010)
2005	*Escherichia coli* O157 VT2	Ensalada IV Gama	>120	Suecia, Noruega	SoderstrÖm y col.(2005)

2005	*Salmonella* typhimurium DT104	Ensalada IV Gama	96	Reino Unido	Health Protection Agency (HPA, 2005)
2005	*Salmonella* typhimurium DT104	Ensalada IV Gama	>60	Suecia, Finlandia	Takkinen y col (2005)
2004	*Salmonella* thompson	Ensalada IV Gama	100	Dinamarca, Noruega, Suecia	Nygard y col (2007)
2004	*Salmonella* newport	Ensalada IV Gama	375	Reino Unido	HPA (2004)
2004	*Yersinia*	Lechuga Iceberg	47	Finlandia	Nuorti y col. (2004)
2003	*Salmonella*	Ensalada IV Gama	40	Reino Unido	HPA (2003)
2002	*Clysclospora cayetanensis*	Ensalada IV Gama con condimentos frescos	34	Alemania	Döller y col. (2002)
2002	*Escherichia coli* O157 PT34 VT2	Ensalada de pepinos	21	Reino Unido, Francia	Duffell y col. (2003)
2001	*Virus Hepatitis* A	Ensalada IV Gama	54	Suecia	Nygard y col. (2001)
2001	*Salmonella* newport PT33	Ensalada IV Gama	19	Reino Unido	Ward y col. (2002)
2001	*Salmonella* typhimurium DT104	Ensalada IV Gama	361	Reino Unido	Horby y col. (2003)

Efectos económicos de los brotes de toxicoinfecciones alimentarias

Cuando ocurre un brote de toxiinfecciones alimentarias, el mercado se ve seriamente afectado y en productos como los vegetales IV Gama, las consecuencias son incalculables porque no sólo se habla de pérdidas económicas, sino también de pérdida de confianza en el sector y por tanto resultando en una caída en el consumo. Un ejemplo fue el brote ocurrido en EE.UU. en el 2006, donde las ventas de ensaladas se redujeron hasta el 70% a lo largo del tiempo en el que el brote estuvo presente en la opinión pública (Todd y otros 2009). Además se requirió un largo periodo de tiempo para recuperar la confianza del consumidor, sin alcanzarse en ningún momento los niveles obtenidos antes de la aparición del brote.

Fuentes de contaminación

En los vegetales IV Gama, la contaminación bacteria puede darse en diversas etapas de la cadena productiva; según factores pre-cosecha, cosecha, pos-cosecha, y consumo, (Beuchat y Ryu, 1997; Delaquis y col., 2007; Doyle y Erickson, 2008; FAO/OMS, 2008b; Hutchison y col., 2008; Izumi y col., 2008; Selma y col., 2007). Según Beuchat (2006) y Mandrell (2009), se han identificado como las causas más frecuentes de contaminación microbiana durante precosecha y cosecha, las siguientes:

- El agua de irrigación contaminada con residuos procedentes de granjas de animales.
- La aplicación de fertilizantes orgánicos de origen animal o humano.
- El contacto directo de animales (salvajes o domésticos) con el producto vegetal fresco cuando está creciendo en el cultivo.
- Las inundaciones por lluvias o aguas de escorrentías. También puede ser una ruta de contaminación potencial si las granjas ganaderas están cerca de los campos de producción de hortalizas.
- La etapa de recolección en el campo, a través del contacto directo con humanos, si no cumplen buenas prácticas agronómicas.

En la etapa de transformación, comercialización y preparación culinaria (poscosecha y consumo), responsabilizan como las causantes de la contaminación del producto a las siguientes prácticas (Gelting y col., 2011; Söderström y col., 2005; Tyrrel y col., 2006):

- El uso de agua de lavado contaminada.
- El uso utensilios no desinfectados
- Deficientes prácticas de manejo higiénico de frutas y hortalizas
- Rotura de la cadena de frio, con elevación dramática de la temperatura

Microorganismos presentes en vegetales IV Gama

Los vegetales de hojas frescos, pueden albergar potenciales patógenos humanos como: *Salmonella*, *Listeria monocytogenes*, *Shigella*, *Clostridium botulinum*, *Escherichia coli*, *Campylobacter*, *Yersinia*, *Vibrio* y *Staphylococcus aureus* (FAO/OMS, 2008a; 2008b; OMS 1998), Cryptosporidium y Virus de hepatitis A, (indicadores de contaminación fecal); que son transmitidos a través de la ingesta de alimentos, (Doyle y Erickson 2006). La contaminación del vegetal puede ocurrir a nivel superficial o en los tejidos internos, que se conoce como la internalización de las bacterias (Ibarra-Sánchez y col., 2004; Moyne y col., 2011). Estos microorganismos una vez en el vegetal son capaces de sobrevivir en diversas superficies, y superar condiciones de estrés y mantenerse latentes hasta conseguir unas condiciones más adecuadas para su crecimiento y colonización del medio. Por ello muchos estudios han enfocado su interés en el comportamiento de los microorganismos en el compost, agua, plantas y su potencial como agente patógeno responsable de comprometer la seguridad microbiológica de los alimentos (Beuchat,

1996; Beuchat y col., 2004; Gleeson y O'Beirne 2005; Harris y otros, 2003). La principal razón de la capacidad de supervivencia observada es que los microorganismos son capaces de adaptarse a condiciones desfavorables, provocando que los métodos de control convencionales dejen de ser efectivos para inhibir la carga microbiana. (Behrsing y col., 2003; Møretrø y col., 2012).

En el desarrollo de esta tesis, nos enfocamos específicamente en *Salmonella* y *Escherichia coli* O157:H7 debido a que en los últimos años han sido las responsables principales de la mayoría de las toxiinfecciones asociadas a vegetales frescos de hojas como puede observarse en la Tabla 1. Por ello, a continuación únicamente se describen estos dos patógenos.

Salmonella spp.

Las bacterias del Género *Salmonella* son Gram negativas y anaerobias facultativas, lo cual les habilita para crecer con bajas concentraciones de oxígeno, como las que se emplean en la conservación de los productos de IV Gama. Su crecimiento se ve reducido por debajo de 15 ºC, y por debajo 7 ºC la mayoría no son capaces de crecer (Carrasco y col., 2012; Francis y col., 1999; Chang y Fang, 2007). Además de estas concidiones de resistencia, *Salmonella* ha sido detectada en muchos tipos de productos vegetales (lechuga, espinacas, etc.) y ha sido asociada a brotes relacionados con el consumo de estos productos (OMS, 1998). Se han identificado como los que afectan con mayor frecuencia al humanos a S. typhimurium y S. enteritidis (Prendergast y col., 2008; Fashae y col., 2010; Hendriksen y col., 2011). Produciendo toxiinfecciones alimentarias, debido a la ingestión de alimentos contaminados. Además, *Salmonella*, es reconocida en todo el mundo como uno de los patógenos más comunes causantes de gastroenteritis (Wegener y col., 2003; Forshell y Wierup, 2006). La enfermedad se caracteriza por un cuadro agudo de fiebre, dolor abdominal, nauseas y vómitos. Las principales vías de trasmisión son a través del consumo de carne de pollo y las carnes rojas contaminadas, leche y sus derivados, frutas, vegetales, huevos, etc. (Caballero-Torres, 2008).

En la Unión Europea, se notificaron 99,020 casos en humanos en el año 2010 (EFSA, 2012). En los Estados Unidos, se estima que cada año enfermen por salmonelosis alrededor de 1,4 millones de personas (CAC, 2005) y según Majowicz y col. (2010), en Asia son responsables de alrededor de 37,600 muertes anuales.

Según los últimos informe de la EFSA, *Salmonella* fue detectada en una baja proporción de muestras de frutas y hortalizas en países de la Unión Europea en 2005, 2006 y 2007 (EFSA, 2006, 2007, 2009). Los productos estudiados fueron en su mayor parte productos pre-cortados listos para usar. En España solo el 0,3 % de las muestras dieron positivo en 2006 (n=896), mientras que no se encontraron muestras positivas en 2007 (n=212). Sin embargo, a pesar de la escasa presencia detectada en los muestreos realizados, en diferentes países de la Unión Europea se han producido brotes causados por *Salmonella* y el consumo de productos vegetales frescos (EFSA, 2007, 2009, Mukhopadhyay y Ramaswany, 2012). Quizás la capacidad de supervivencia en vegetales de hasta 4 semanas observada por algunos estudios (Dawson y col., 2005), es un elemento que debería ser considerado para explicar la aparición de brotes a pesar de su baja incidencia.

Escherichia coli

El microorganismo *Escherichia coli* es Gram negativos y anaerobio facultativo, lo cual

les pertite crecer a bajas concentraciones de oxígeno que son habituales en el tracto gastrointestinal del hombre y la mayoría de los animales (Olivera y col., 2012). *E. coli* según su mecanismo de patogénesis se ha clasificado en diferentes grupos (Donnenberg y Whittam, 2001; Robins-Browne y Hartland, 2002). Así, se proponen 5 grupos principales: *E. coli* Enterotoxigénica (ETEC), *E. coli* Enteroinvasiva (EIEC), E. coli Enteroagregativa (EAEC), *E. coli* Enteropatogénica (EPEC) *E. coli* Enterohemorrágica (EHEC) (Bugarel y col., 2011):

- ETEC: Las cepas Enterotoxigénicas como su nombre lo indica producen toxinas termoestables y/o termolábiles que actúan en el intestino delgado. Son muy conocidas por producir la diarrea del viajero. Su período de incubación oscila entre 8–44h con una media de 26h y se caracteriza por síntomas como nauseas, dolor abdominal moderado y diarrea, tras la ingestión de agua y alimentos contaminados. Los serogrupos más comunes incluyen O6, O8, O15, O20, O25, O27, O63, O78, O80, O114, O115, O128AC, O148, O153, O159 y O167, entre otros (Mossel y col., 2003).
- EIEC: Producen una enfermedad más grave, a menudo acompañada de diarreas sanguinolentas (disentería). Son bacilos inmóviles y la mayoría son anaerobias, así como fermentadoras tardías de la lactosa. Los serogrupos principales son O28ac, O29, O112, O124, O136, O143, O144, O152, O164 y O167.
- EAEC: Están relacionadas con fenómenos de diarrea profusa en bebés y niños. No forman enterotoxinas, pero se diferencian del resto de *E. coli* por un característico patrón de adherencia a las células del epitelio intestinal, mediante fimbrias, de forma agregativa en las célula HEp-2 (Caballero-Torres, 2008; Montville y Matthews, 2009).
- EPEC: son la principal causa de diarrea infantil en países desarrollados (Gomes y col., 1989, 1991; Nataro y Kaper, 1998). Rowe y col. (1974) identificaron el serogrupo O158 tras un brote de enteritis en niños originado en una guardería. Éstas se caracterizan por producir diarreas acuosas que pueden llegar a ser profusas, acompañadas de fiebre, malestar general y vómitos (Levine, 1987); asimismo provocan la destrucción de las micro-vellosidades tras su adherencia y esfacelamiento (A/E) a la mucosa intestinal (Caballero-Torres, 2008; Montville y Matthews, 2009). El hombre es el principal reservorio de EPEC y los serogrupos que más le afectan son O26, O55, O86, O111, O114, O119, O125, O126, O127, O128a y b, O142 y el O158.
- EHEC: Este serogrupo está implicado con numerosos brotes de toxiinfecciones alimentarias en muchos países desarrollados y se caracteriza por producir procesos infecciosos que ocasionan complicaciones severas como colitis hemorrágica y espasmos abdominales. Estas cepas producen potentes citotoxinas como las toxinas shiga (Stx), llamadas así por su relación con la toxina de Shigella dysenteriae, y las verotoxinas. Su factor de virulencia está determinado por la producción de factores de adherencia, de citotoxinas y enterohemolisinas, y la capacidad para transportar hierro y producir lesiones de adherencia y esfacelamiento con destrucción de las microvellosidades del epitelio intestinal. Actualmente, el serotipo más peligroso es *E. coli* O157:H7 y O157:H– que están muy asociados a enfermedades de origen alimentario. Sin embargo, a nivel mundial la prevalencia de infecciones por otros serogrupos de EHEC no O157, están siendo subestimadas ya que los casos por EHEC no O157:H7 son muy frecuentes (Montville y Matthews, 2009).

En el año 2010, el serotipo O157:H7 fue responsable de 4,000 casos de toxiinfecciones alimentarias en la Unión Europea, lo cual representó un 12% más de las producidas en el año 2009; donde los niños de 0–4 años (1,161 casos) y de 5–14 años (>40 casos) fueron los más afectados (EFSA, 2012).

Si bien, la mayoría de las cepas de *E. coli* no son consideradas agentes patógenos, algunas sí actúan como patógenos oportunistas causando considerables daños sobre todo en personas inmunodeprimidas, niños o ancianos (Gassama y col., 2001).

Ademas, la presencia de *E. coli* en alimentos crudos es considerado un indicador directo (aquella que se produce durante el procesado) o indirecto (aquella que ocurre a través del agua de lavado o las aguas residuales) de contaminación fecal (Renata, 2010); también, la presencia de *E. coli* en los alimentos y el agua fue aceptada como signo manifiesto de la posible presencia de patógenos oportunistas.

En el ámbito de la Unión Europea se han atribuido brotes recientes causados por EHEC del serotipo O157:H7 al consumo de productos vegetales frescos contaminados (Söderström et ál., 2005; Takkinen et ál., 2005; Friesema et ál., 2007), sin embargo, la presencia de EHEC en estos productos dentro de la Unión Europea parece ser muy baja, ya que no se encontraron muestras positivas en 2005 (n=493), 2006 (n=1126), ni en 2007 (n=2083) (EFSA, 2006, 2007a, 2009).

Tratamientos de Higienización en vegetales IV Gama

Mayoritariamente el proceso de higienización en los vegetales IV Gama se realiza a través del lavado de la materia prima fresca con productos de diversos orígenes, pero también se puede dar la higienización a través de la aplicación de técnicas físicas o mecánicas; que incrementan la eliminación de la suciedad, de la materia orgánica y de la flora bacteriana.

En la Tabla 2, se encuentra recogidos los métodos más utilizados en la higienización de vegetales, los cuales hemos clasificado en: físicos, químicos y aquellos basados en antimicrobianos naturales. El tratamiento de higienización en vegetales IV Gama más estudiado es, en primer lugar, el cloro, seguido por el ozono y el agua electrolizada. En esta tesis algunos de estos métodos fueron objeto de estudio.

La formación de subproductos potencialmente peligrosos, la falta de eficacia y la dependencia de factores externos sobre la acción del cloro, son los motivos que han llevado a la búsqueda de alternativas al uso del cloro. Sin embargo los resultados obtenidos hasta la fecha con los métodos alternativos no han sido muy alentadores en comparación a los obtenidos con el cloro. Estos métodos alternativos incluyen: el ozono, agua electrolizada, ácidos, ácidos orgánicos, peroxiacético, compuestos fenólicos, bacteriocinas, peróxido de Hidrógeno, permeado de suero lácteo, ultrasonidos, aceites esenciales, etc. (Tabla 2). Asimismo, se ha propuesto el uso combinado de diferentes higienizantes para aumentar su capacidad desinfectante respecto a su uso individual (McWatters y col., 2002; Beltrán y col., 2005b). Entre ellos podemos destacar el uso de: ozono + cloro (Garcia y col., 2003), luz ultravioleta + peróxido de hidrógeno (Xie y col., 2008), peróxido de hidrógeno + ácido láctico (Lin y col., 2002), ozono + ácido peroxiacético (Beltrán y col., 2005b.)

Cloro

El Cloro es el higienizante o desinfectante más usado por la industria de IV Gama, en sus diversas formas y presentaciones, como: hipoclorito de sodio (NaClO), hipoclorito

de calcio ($Ca(ClO)_2$), cloro gas (Cl_2) ácido hipocloroso ($HClO^-$), El dióxido de cloro (ClO2) gas y líquido, (Cantwell y Suslow, 2002; Sapers, 2001; Suslow, 1997; Varoquaux y Mazollier, 2002). El hipoclorito de sodio (NaClO), por su facilidad de uso, su bajo coste, y su relativa eficacia (Luo y col., 2012), es el más utilizado en las industrias de frutas y hortalizas (Al-Haq y col., 2005) en concentraciones de 50-100 mg/L, con un tiempo de contacto de 1-2 minutos (FAO/OMS, 2008b). No obstante, en algunos casos las concentraciones utilizadas de cloro pueden alcanzar hasta 200 mg / L, utilizándose tiempos similares de contacto a los indicados anteriormente (Adams y col., 1989; Beuchat, 1998). El efecto antimicrobiano de hipoclorito de sodio depende de la cantidad de cloro libre (en forma de ácido hipocloroso, $HClO^-$) presente en el agua que entra en contacto con las células microbianas (Bartz y col., 2001). Su efecto radica en la interrupción de la síntesis de proteínas, la oxidación de la glucosa y de ciertas enzimas en el metabolismo de carbohidratos, reacciones con ácidos nucleicos, purinas, pirimidinas, la descarboxilación oxidativa de los aminoácidos, las lesiones en la estructura del ADN, y en general un desequilibro celular que desencadena en problemas de absorción de oxígeno (Dukan y col., 1999; Hricova y col., 2008; Mariott y Gravani, 2006; McDonnell y Russell, 1999).

El cloro libre se consume en contacto con la materia orgánica y residuos de alimentos, y la eficacia del mismo depende de la disociacion de $HClO^-$ con el pH (Beuchat, 1998; Delaquis y col., 2004; Fatemi y Frank, 1999; Gonzalez y col., 2004; Hilgren y col., 2007; Klaiber y col., 2004; Ruiz-Cruz y col., 2007a,b). Además, se pierde su actividad con la exposición al aire, a la luz y a los metales. Existen factores adicionales que influyen sobre la eficacia del tratamiento con hipoclorito como son la duración y temperatura de tratamiento y los componentes de tejidos vegetales (Beuchat, 1998; Hilgren y col., 2007). Por medio de una metodología de superficie de respuesta, Lu y col., (2007) concluyeron que la eficacia de lavado de hipoclorito en la reducción de bacterias aerobias mesófilas presentes en lechuga fresca cortada fue influenciada en gran medida por la concentración utilizada, moderadamente por la duración del tratamiento y en menor medida por la relación agua-lechuga.´

El pH recomendado en la desinfección de cloro es 6,5 a 7,5, para evitar corrosión y mantener la eficacia. En estas condiciones encontramos entre un 50 y un 95 % del cloro en forma de $HClO^-$que posee mayor eficacia antimicrobiana que la forma ClO^-. Si el pH se reduce a niveles inferiores a los recomendados, se pierde cloro al liberarse en forma Cl_2. Cuando se trabaja a temperaturas altas, también se pierde cloro ya que aumenta la volatilidad del Cl_2, aun cuando se aumenta la eficacia del cloro. Para un control adecuado de la desinfección con cloro, hay dos opciones, por un lado está la posibilidad de controlar el pH y la concentración de HClO y ClO^-, y por otro el control del potencial oxido-reducción. Aunque este última opción no es aceptada por unanimidad como un indicador del cloro disponible. A todos estos parámetros que determinan la eficacia se añade, como un aspecto negativo del uso del cloro, la preocupación por sus efectos nocivos sobre la salud, como es irritación en la piel y en el tracto respiratorio que se producen como consecuencia de una exposición prolongada al vapor de cloro generado en la desinfección (Abadias y col., 2008).

Tabla 2. Agentes higienizantes utilizados en vegetales de hojas durante el procesamiento de alimentos IV Gama.

Físicos	
Intensos pulsos de luz	Gómez-López y col., (2005 a y b)
Irradiación ionizante	Fan y Sokorai (2008); Mintier y Foley (2006); Mañas y Pagán (2005); Niemira (2008).
La alta presión hidrostática	Mañas y Pagán (2005)
Luz UV-C	Allende y col., (2006); Artes (2009); Cho y col., (2010); Guerrero-Beltran y Barbosa (2004); Hadjok y col., (2008);
Pulsos Eléctricos	Mañas y Pagán (2005)
Tratamientos térmicos suaves	Alegria y col., (2010); Alegria y col., (2009); Baur y col., (2005); Rahman y col., (2011).Selma y col., (2008).
Ultrasonido	Guerrero y col., (2005); Huang y col., (2006); Joyce y col., (2003); Piyasena y col., (2003); Seymour y col., (2002).
Vapor	Martín-Diana y col., (2007)
Vapor sobrecalentado	Cenkowski y col., (2007)
Mecánico (agua de grifo)	Caldwell y col., (2003); Singh y col., (2002 a y b); Workneh y col., (2007).
Químicos	
Ácido acético	Huang y col., (2012); Chang y Fang (2007); Nascimento y col., (2003); Samara y Koutsoumanis (2009).
Ácido ascórbico	Akbas y Ölmez (2007); Artés y col., (2009); Francis y O´Beirne (2002).
Ácido cítrico	Akbas y Ölmez (2007); Allende y col., (2009); Artés y col., (2009); Francis y O´Beirne (2002); Ihl y col., (2003); Rahman y col., (2011); Samara y Koutsoumanis (2009).
Ácido láctico	Akbas y Ölmez (2007); Allende y col., (2008b); Huang y col., (2012); Lin y col., (2005); Lopez-Galvez y col., (2009); Samara y Koutsoumanis (2009); Smigic y col., (2009); Velazquez y col., (2009).
Ácido octanoico	Hilgren y Salverda (2000)
Ácido organico	Akbas y Ölmez (2007); Huang y Chen (2011); Lopez-Galvez y col., (2009); Yuk y col., (2006).
Ácido peroxiacético	Allende y col., (2008b); Allwood y col., (2004);Beuchat y col., (2004); Hilgren y Salverda (2000); Lopez-

	Galvez y col., (2009); Silveira y col., (2008); Zhang y col., (2009).
Agua Electrolizada	Abadias y col., (2008); Al-Haq y col., (2005); Gómez-López y col., (2013); Issa-Zacharia y col., (2011); Joyce y col., (2003); Rahman y col., (2011); Smigic y col., (2009); Stopforth y col., (2008); Zhang y col., (2011).
Clorito sódico acidificado	Martínez-Sánchez y col., (2006); Ruiz-Cruz y col., (2007a,b)
Cloro (hipoclorito de sodio, ácido hipocloroso)	Akbasy Ölmez (2007); Allende y col., (2008a,b; 2009); Allwood y col., (2004); Behrsing y col., 2000; Beltrán y col., (2005b); Beuchat y col., (2004); Beuchat y col., (2004); Chang y col., (2000); Foley y col., (2002); Francis y O´Beirne (2002); Issa-Zacharia y col., (2011); López-Gálvez y col., (2009); López-Gálvez y col., (2010); Luo (2007); Mckellar y Lu., (2004); Niemira (2008); Pirovani y col., (2004); Rodgers y col., (2004); Stopforth y col., (2008); Workneh y col., (2007); Zhang y col., (2009).
Dióxido de cloro (gas y líquido)	Ayyildiz y col., (2009); Huang y col., (2006); Keskinen y col., (2009); Kim y col., (2006); Lee y Back, (2008); Mahmoud y Linton, (2008); Rodgers y col., (2004); Silveira y col., (2008); Singh y col., (2002 y 2003); Smigic y col., (2009); Zhang y Faber, (1996).
Ozono	Beltrán y col., (2005a; b); Garcia y col., (2003); Hadjok y col., (2008); Lin y col., (2002); Akbas y Ölmez (2007); Selma y col., (2006, 2007 y 2008); Singh y col., (2002b); Yuk y col., (2006). Zhang y col., 2005.
Peróxido de hidrógeno	Allwood y col., (2004); Hadjuk y Surówka (2005); Huang y Chen, (2011); Huang y col., (2012); Lin y col., (2002); Silveira y col.; (2008).
Antimicrobianos naturales	
Aceite esencial	Karagozlu y col., (2011); Singh y col., (2003); Uyttendaele y col., (2004).
Chitosan	Guerrero y col., (2005)
Dicloroisocianurato sódico	Nascimento y col., (2003)
Extracto de ajo	Ihl y col., (2003)
Flavonoides	Allende y col., (2008b)
Lactoperoxidasa -tiocianato	Allende y col., (2008b)
Nisina (+ EDTA)	Silveira y col., (2008)
Permeato de suero	Martín-Diana y col., (2006)
Vinagre	Nascimento y col., (2003)

El uso de hipoclorito de sodio también se ha asociado con la formación de carcinógenos a partir de subproductos clorados tales como cloraminas y trihalometanos, ácidos haloacéticos (AHAs) y haloacetonitrilos (HANs) (Alegria y col., 2009; OMS, 2000; Richardson y col., 2002 y 2007; kitis y col., 2010). No obstante se ha demostrado que el lavado de productos de IV Gama con cloro no supone un riesgo de exposición a estos subproductos potencialmente peligrosos por parte de los consumidores según estudios realizados según la ingesta de alimentos desinfectados con productos a base de cloro (FAO/OMS, 2009).

El hipoclorito de sodio ha sido extensamente estudiado por su eficacia para inactivar los patógenos bacterianos en las frutas y verduras, incluyendo *L. monocytogenes*, *Salmonella* typhimurium, *Escherichia coli* O157: H7; en: lechuga, pimientos, melones, manzanas, tomates, zanahorias cortadas frescas, zanahoria rallada (Alvarado-Casillas y col., 2007; Beuchat y col., 1998; Beuchat y col., 2004; González y col., 2004; Ruiz-Cruz y col., 2007a; Weissinger y col., 2000; Zhuang y col., 1995.) Aunque, en menor proporción existen estudios del higienizante sobre la flora natural (Nascimento y col., 2003) y sobre la posterior recuperación de los patógenos, durante el almacenamiento, distribución y consumo (Akbas y Ölmez, 2007; Delaquis y col., 2004).

El cloro además es el desinfectante más frecuentemente usado como referencia para evaluar y comparar la eficiencia de otros productos o procesos de higienización. Por ejemplo, Adams y col. (1989) compararon el uso del cloro en lechuga lavada con 0-300 mg / L de cloro libre sin ajuste de pH y el producto sin lavar, donde obtuvo que con cloro (100-200 mg / L) causó un aproximado de 0,9 a 1,2 log disminución en el recuento de aerobios en placa de lechuga fresca cortada. En todo caso, las comparaciones entre estudios son difíciles ya que numerosos factores varían de unos ensayos a otros (Beuchat y col., 2003; FAO/OMS, 2008b).

Hasta la actualidad en el mejor de los casos la eficacia del cloro no es capaz de superar los 3 log de reducción, independientemente del producto. (Doyle y Erickson, 2008; García y col., 2003; Klaiber y col., 2005; Ruiz-Cruz y col., 2007b). Además, la desinfección de los productos de IV Gama con cloro a las concentraciones y tiempos de contacto indicados no han demostrado ser mucho más efectiva en el lavado con agua sin desinfectantes, donde actúa una eliminación mecánica por arrastre.

Agua electrolizada

El agua electrolizada (AE) es el producto de la electrólisis del agua de grifo o una solución diluida de NaCl o KCl $MgCl_2$ en una célula de electrólisis. En otras palabras, AE se obtiene al hacer pasar una corriente eléctrica por agua a la que se ha añadido cloruro sódico. El agua se electroliza, obteniéndose dos tipos de agua que pueden permanecer separados por una membrana, o mezclarse si no hay separación. En el cátodo se forma agua electrolizada básica con alto poder reductor, y en el ánodo agua electrolizada ácida, con poder oxidante y mayor capacidad bactericida. Es considerada como una de las alternativas más prometedoras para sustituir al cloro ya que es una de las tecnologías más innovadoras de descontaminación a la vez que respetuosa con el medio ambiente en sus tres presentaciones: oxidante, neutra y ácida (Abadias y col., 2008; Huang y col., 2008).

El AE ácida tiene efecto antimicrobiano debido a que contiene cloro gas, ión hipoclorito, ácido hipocloroso, ozono y radicales (O^-, OH^-, y Cl^-), presentando un elevado potencial de oxido-reducción. A pH bajo, la forma mayoritaria y más efectiva es el $HClO^-$ (Al-Haq y col., 2005). La desventaja del AE ácida es su bajo pH (<3), que

puede ser corrosivo para el equipamiento, peligroso para los operarios por la liberación de Cl_2, y dañino para el producto vegetal (Guentzel y col., 2008). El agua electrolizada básica se usa normalmente como limpiador o como pre-tratamiento antes del lavado con agua electrolizada ácida (Hricova y col., 2008). El AE neutra se obtiene mezclando AE ácida con iones OH^-, o generando el agua electrolizada sin separación mediante membrana (Hricova y col., 2008). El AE neutra con pH 6,5 contiene HClO (95 %), ClO^- (5 %) y trazas de Cl_2 (Guentzel y col., 2008). El AE ácida y neutra ha dado buenos resultados en productos de IV Gama (Abadias y col., 2008b; Gómez-López y col., 2008; Hricova y col., 2008; Huang y col., 2008), así como para el mantenimiento de la calidad microbiológica del agua de proceso evitando así la contaminación cruzada (Ongeng y col., 2006). Una de las ventajas del agua electrolizada es que sólo requiere de NaCl y agua para su generación, por lo que tras la inversión inicial, el gasto que conlleva su uso es mínimo (Al-Haq y col., 2005; Hricova y col., 2008). Además, su eficacia es mayor que la del hipoclorito a igual concentración de cloro libre (Abadías y col., 2008b). Las desventajas la necesidad de generar constantemente agua electrolizada de forma continua debido a que su estabilidad no está muy estudiada a lo largo del tiempo. También presenta la formación de subproductos y la baja eficacia en presencia de materia orgánica similar a lo que ocurre en el cloro (Kiura y col., 2002; Park y col., 2008; 2009). Otra desventaja es que su almacenamiento reduce su poder bacteriocidaa lo largo del tiempo ya que no está estabilizada como ocurre con las soluciones de hipoclorito (Huang y col., 2008).

Ozono

El ozono (O_3) se genera al someter las moléculas de oxígeno a descarga eléctrica de alto voltaje (Khadre y col., 2001), en consecuencia se convierte en un potente oxidante, y muy eficaz en la desinfección de agua (Parish y col., 2003). La capacidad higienizante es similar al cloro, siendo capaz de reducir significativamente flora natural y patógena y manteniendo la calidad del producto, siendo esta característica muy importante en la industria de vegetales de hoja (Rodgers y col., 2004; Koseki e Isobe, 2006; Hassenber y col., 2007; Selma y col., 2007a; Martínez-Sánchez y col., 2008; Ölmez y Akbas, 2009b). En este sentido, Beltrán y col. (2005a) aseguran que el ozono puede prolongar aún más la vida útil respecto al cloro. En general, esta tecnología no resulta en subproductos peligrosos para la salud, excepto en presencia de bromuro (Richardson y col., 2000; Hua y Reckhow, 2007; Ölmez y Kretzschmar, 2009a). Entre las desventajas más importantes destacamos: elevada inversión inicial, alto poder de corrosión, forma grandes cantidades de espuma y elevada dificultad para controlar la concentración activa en el agua de lavado (Suslow, 1997; Parish y col., 2003; Selma y col., 2007a).

Ácido peroxiacético

El ácido peroxiacético (CH_3CO_3H) es un oxidante fuerte, generado tras reaccionar el ácido acético con peróxido de hidrógeno (Wagner y col., 2002; Dell'Erba y col., 2007).Presenta mayor potencial de oxidación que el cloro pero menor que el ozono y su mecanismo de acción se basa en la oxidación de componentes celulares (lípidos, proteínas y ácidos nucleicos) (McDonnell y Russell, 1999).

Entre las ventajas se encuentran, que no requiere grandes inversiones económicas, es eficaz en presencia de materia orgánica, no resulta en subproductos peligrosos, escasa dependencia del pH, rapidez de actuación y es efectivo en el agua de lavado (Beuchat y col., 2004; Kitis, 2004; Wang y col., 2006; Ruiz-Cruz y col., 2007a). El efecto sobre la calidad del producto depende de la concentración usada y del tipo de producto

(Vandekinderen y col., 2008). En las desventajas se destaca el incremento en la carga de materia orgánica del agua y la necesidad de añadir un agente estabilizante ya que es inestable (Kitis, M., 2004).

Ácidos orgánicos

Los ácidos orgánicos han sido ampliamente aplicados como conservantes en diferentes alimentos; sin embargo su aplicación como higienizante ha sido más reciente (Parish y col., 2003). Como conservante, actúa alterando la permeabilidad y el transporte en la membrana celular, a la vez que reduce el pH intracelular lo que afecta al metabolismo de la célula (Kreske, 2008). En las ventajas destaca que permite reducir la carga microbiana sin necesidad de implantación de equipamiento especifico. Como desventajas, necesita un prolongado tiempo de contacto y su adición incrementa la carga orgánica en el agua de proceso (Ölmez y Kretzschmar, 2009). Por otro lado, su efecto sobre la calidad del producto no es concluyente, ya que existen trabajos que han detectado deterioro evidente en la calidad del producto (Wu y col., 2000; Vijayakumar y Wolf-Hall, 2002; Chang y Fang, 2007), en contraposición a otras investigaciones que han obtenido una mejora en la calidad del producto (Kim y Klieber, 1997; Aguayo y col., 2003; Akbas y Ölmez, 2007).

Microbiologia Predictiva

La microbiología predictiva es una rama especializada de la microbiología de los alimentos, dedicada a estudiar y predecir el comportamiento microbiano frente a factores ambientales e intrínsecos al microorganismo, haciendo uso para tal fin, de funciones matemáticas (McMeekin y col., 1993). Estas funciones representan la respuesta de los microorganismos como resultado de la interacción de diferentes factores (temperatura, pH, actividad de agua etc.). El proceso por el cual se obtienen estas funciones es conocido como modelización (Ratkowsky y col., 1982; Roberts and Jarvis, 1983). La modelizacion es el uso de ecuaciones matemáticas que emplean leyes físicas y químicas para describir, en términos matemáticos, el comportamiento de un sistema real (Dym, 2004).

Dentro de los modelos existen diferentes clasificaciones: según el tipo de respuesta bacteriana, según el origen modelo, según el nivel de descripción y desarrollo y por último, según el tipo de ecuación matemática utilizada como base en su construcción. Una clasificación más general de los diferentes modelos existentes es aquella que los divide en modelos mecanicistas y empíricos (Buchanan y col., 1997). Los modelos mecanicistas son aquellos modelos que han sido desarrollados sobre la base de varias hipótesis teóricas y que están relacionados con el conocimiento de los procesos implicados en la cinética del comportamiento de los bacteriano o conocido también, como el mecanismo de acción (McLauchlin y col., 2004). Mientras que los modelos empíricos son aquellos desarrollados experimentalmente y sin considerar previamente el micanismo de acción: físicos, fisiológico y bioquímico. Las funciones log-logística o la ecuación modificada de Gompertz son ejemplos de modelos de crecimiento de carácter empírico (Haas y col., 1999; Buchanan y col., 1997). Debido a la dificultad de desarrollar modelos mecanicistas como consecuencia del conocimiento limitado que se tiene sobre los procesos celulares y bioquímicos, la mayoría de los modelos tienden a ser de carácter cuasi-mecanicistas, donde se introducen parámetros con significación biológica como es el caso del modelo de Baranyi y Roberts (1994) donde el tiempo de latencia es definido sobre la base de la concentración de una sustancia X que es

limitante para el comienzo del crecimiento (Baranyi y Roberts, 1994)

Dependiendo del nivel de descripción y desarrollo los modelos cinéticos pueden ser clasificados como primarios, secundarios o terciarios. A continuación realizaremos una descripción de los modelos más usados en microbiología predictivos basado sobre esta clasificación.

Modelos primarios

Los modelos primarios son aquellos que describen a través de una ecuación matemática el cambio de concentración microbiana con respecto al tiempo (Whiting y Buchanan, 1994). Estos son mayoritariamente modelos cinéticos donde se describe los fenómenos de crecimiento y muerte celular. A continuación presentamos una clasificación de los modelos cinéticos primarios que han tenido un mayor desarrollo en los últimos años (McKellar y Lu, 2004):

1. Modelos de Crecimiento.
2. Modelos de Inactivación.
3. Modelos de Supervivencia.
4. Modelos de Crecimiento/no crecimiento y de probabilidad.

También se pueden considerar en este grupo de modelos primarios, modelos no cinéticos, relacionados con fenómenos de transferencia o contaminación cruzada. Estos representan procesos físicos en contraste a los modelos cinéticos donde los mecanismos implicados son principalmente de índole biocinética (Perez-Rodriguez y col., 2008).

Los modelos de crecimiento se generan en un rango de condiciones ambientales que permiten el crecimiento microbiano, el cual, se identifica con el incremento en la población bacteriana en el transcurso del tiempo. Los microorganismos cuando crecen en un sistema cerrado donde los nutrientes están limitados desarrollan curvas sigmoidales (Peleg, 2006a) del tipo representado en Figura 4. Cada una de las fases observadas en la curva de crecimiento se define a través de un parámetro matemático, y a los cuales llamamos parámetros cinéticos (Zwietering, 1990):

- El tiempo de latencia o adaptación (A).
- La tasa de crecimiento (B).
- Densidad máxima de población (C).
- Fase de muerte o decaimiento (D)

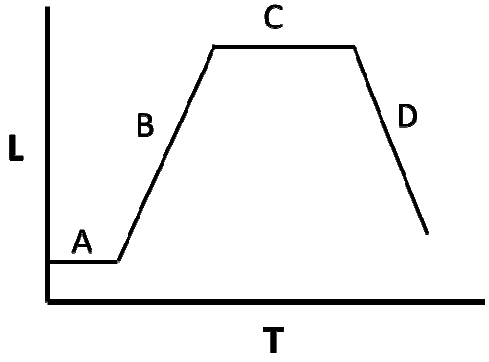


Figura 4. Representación esquemática de las fases sufridas por las bacterias en un sistema cerrado donde existe un agotamiento de los recursos. El eje L representa concentración de la bacteria y T el tiempo, ambos en unidades relativas.

En los **modelos de crecimiento primarios**, las funciones matemáticas más utilizadas son la ecuación modificada de Gompertz, Baranyi y Roberts y logística (Gibson y col., 1988; McMeekin y col., 1993b; Baranyi y Roberts, 1994) (Table 3). Las dos primeras ecuaciones describen un perfil sigmoidal sobre valores en la escala logarítmica (Gibson, 1987). En cambio, la ecuación logística presenta un perfil no sigmoidal dado que es el resultado del desarrollo de la ecuación diferencial que describe la fase (B) (Zwitering y col., 1990).

Tabla 3. Modelos de crecimiento primarios

Modelo	Función matemática
Exponencial	$\log(n) = \log(n_0) - \mu_{max} t$
Baranyi y Roberts	$n(t) = n_0 + \mu_{max} A(t) - \frac{1}{m} \ln\left(1 + \frac{\exp(m\mu_{max} A(t) - 1)}{\exp(m n_{max} - n_0)}\right)$
Ecuación modificada de Gompertz	$\log(n) = A + C \exp\left[-\exp\left(-B(t - M)\right)\right]$
Lag+exponencial	$\log(n) = \log(n_0) - \mu_{max}(t - \lambda)$

Donde:

- Para el modelo exponencial n= ufc/g a tiempo t; np= ufc/g a tiempo t=0; t = tiempo (h); µmax = tasa máxima de crecimiento (h-1)
- Para el modelo Baranyi y Roberts m =representa la curvatura existente entre la fase exponencial y la estacionaria; nmax= población máxima (ufc/g);

$$A(t) = t + \frac{1}{\mu_{max}} \ln\left(\frac{e^{-\mu_{max}(t)} + \left(e^{\lambda\mu_{max}\cdot 1}\right)^{-1}}{1 + \left(e^{\lambda\mu_{max}\cdot 1}\right)^{-1}}\right)$$

- Para el modelo de Gompertz B = tasa máxima de crecimiento (h-1); M= el tiempo al cual ocurre la tasa máxima de crecimiento (h); A = máxima asíntota de log10 ufc/g; C = la diferencia entre A y la asíntota inferior de log10 ufc/g
- Para el modelo Lag+exponencial λ= tiempo de latencia lag (h)

Los ***modelos de inactivación*** son aquellos modelos matemáticos que describen la reducción de la población microbiana en función del tiempo. A través de ellos se estima la tasa de muerte microbiana. Normalmente son aplicados para describir el efecto de diferentes tratamientos físicos: térmicos, irradiación, altas presiones (Peleg, 2006b; McKellar y Lu, 2004). Las curvas de inactivación pueden presentar el perfil mostrado en Figura 5, con tres fases principales, hombro, muerte, y cola o asíntota. No obstante, dependiendo de las condiciones, ambientales, experimentales, tipo de microorganismo y alimento, ciertas fases pueden estar ausentes.

- Fase de adaptación “Hombro” (A).
- Fase de Inactivación o muerte (B).
- Fase asintótica “cola” (C)

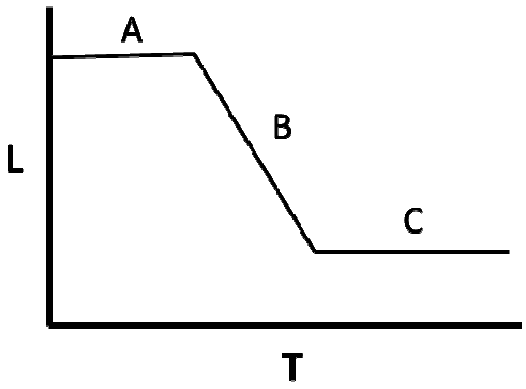


Figura 5. Representación esquemática de las fases sufridas por las bacterias en un sistema cerrado donde existe un proceso de reducción-muerte de la concentración bacteriana. El eje L representa concentración de la bacteria y T el tiempo, ambos en unidades relativas.

Los modelos de inactivación pueden clasificarse en:

- ✓ Modelos lineales, que representan destrucción de la población microbiana siguiendo una cinética de primer orden (fase B en Figura 5). Este modelo ha sido considerado hasta la actualidad como la base para el desarrollo y aplicación de los tratamientos térmicos en alimentos, dando lugar a los conceptos **D** y **z** (Bigelow y Esty, 1920; Mazzota, 2001; Murphy y col., 2002; Van Asselt y Zwietering, 2006) (Tabla 4).
- ✓ Modelos no lineales, que representan la heterogeneidad de la población microbiana en cuanto a su sensibilidad a los tratamientos de inactivación. Se han propuesto diferentes funciones matemáticas entre las que destacamos los modelos bifásicos (Cerf, 1977), que representan dos fases, una inicial de muerte lineal (fase B en Figura 5) y una fase de resistencia representada por una línea asintótica en el modelo (fase C en Figura 5). Estos modelos también pueden contemplar la existencia de un hombro (fase A en Figura 5), que representa una fase inicial de resistencia la tratamiento o simplemente un fase donde el efecto del tratamiento no es completo. En los últimos años se ha propuesto la función de Weibull definida por los parámetros *a* y *b* para representar diferentes patrones de inactivación, siendo una alternativa plausible a la cinética clásica de inactivación (Peleg, 2006a,b). También las funciones logística y de Gompertz; pueden ser aplicadas para describir modelos primarios de inactivación no lineales (Legan y col., 2002) (Tabla 4).

Los ***modelos de supervivencia*** describen más específicamente la inactivación no térmica en condiciones no extremas y que se encuentran cerca de los límites de crecimiento. En cuanto a los tratamientos térmicos, los procesos de supervivencia son observados en un rango de 50-60 ºC. Otros fenómenos que dan lugar a curvas de supervivencia son la desecación en superficies y la exposición a sustancias inhibidoras, tóxicas y conservantes alimentarios. En relación al tratamiento de los datos, estos siguen procesos similares a los aplicados a los modelos de inactivación. Los microorganismos en estos casos suelen presentar fases tipo hombro y cola, que no pueden ser descritas convenientemente por el modelo clásico de inactivación de primer orden (i.e. log-lineal) (Bigelow y Esty, 1920). Por tanto son más propicios los modelos bifásico o trifásicos o bien las diferentes variantes a la función de Weibull. La aplicación de los modelos de supervivencia en la ECRM es clave ya que es capaz de explicar mejor lo que podría ocurrir en una posible recontaminacion, ya que el número de células

transferibles depende en gran medida de la cantidad de células capaces de sobrevivir frente al estrés ambiental (Perez-Rodriguez y col. 2008; Baker, 1993; Jay, 1992, Doyle, 2001; Legan y col., 2002).

Tabla 4. Modelos de inactivación primarios

Modelo	Función matemática
Exponencial (modelo lineal)	$\log(n) = \log(n_0) - kt$
Logístico	$\log(n) = \log(n_0) + \frac{a}{1+\exp(b-ct)}$
Modelo de Weibull	$s = \exp(-bt^n)\ o\ \log(s) = -bt^n$
Lag+exponencial	$L(t) = A + C\exp\left[-\exp(-B(t-M))\right]$

Donde:

- Para el modelo lineal *n*= recuento/g a tiempo *t; t* = tiempo; *k* = una constante de tasa; También: 1/*k* = *D* = tiempo necesario para alcanzar una reducción decimal ufc/g.
- Para el modelo logístico *n* = recuento/g; n_0= recuento/g cuando *t*= cero; *t* = tiempo (horas); *a*, *b* y *c* son los parámetros de ajuste; *t* = tiempo (horas).
- Para el modelo de Weibull *s* = fracción de supervivencia (n/n_0) a tiempo t; *b* = parámetro de escala; *n* = parámetro de forma
- Para el modelo lag+exponencial *L*(t) = $\log_{10}$recuento bacteriano a tiempo *t*; *B* = tasa relativa de muerte máxima(h^{-1}); *M*= el tiempo al cual ocurre la tasa de muerte (h); *A* = máxima asíntota de $\log_{10}$recuento bacteriano C = la diferencia entre A y la asuntota inferior de $\log_{10}$recuento bacteriano.

Los ***Modelos de crecimiento y no crecimiento*** son aquellos modelos que definen los límites que permiten o no el crecimiento del microorganismo en relación a ciertos factores externos como pH, a_w, ácido cítrico, etc., siendo el más significativo la temperatura. Existen variantes de estos modelos que permiten estimar el tiempo necesario para que se produzca crecimiento. Para ello, utilizan datos cualitativos de crecimiento/no crecimiento en diferentes intervalos de tiempo, aplicando técnicas de regresión en la generación de la función matemática (Leistner y col., 1985, Gorris, 2000). Los modelos de probabilidad son modelos que proporcionan la probabilidad de crecimiento a unas condiciones dadas. La utilización de probabilidades requiere el desarrollado de múltiples repeticiones (> 8) para cada una de las combinaciones de factores ambientales estudiados. Este tipo de modelos pueden utilizarse en la ECRM como complemento a los modelos de crecimiento con el objetivo de predecir la existencia de crecimiento o no en cada escenario simulado en el umbral de crecimiento (Genigeorgis, 1981; Gibson y col., 1987, Zhao y col., 2000).

Los ***Modelos de transferencia*** son aquellos modelos que describen la contaminación cruzada entre el entorno y el alimento, además este tipo de modelos son los últimos propuestos en la microbiología predictiva (Perez-Rodriguez y col. 2008; Van Asselt y col., 2006). La contaminación cruzada siempre han sido una causa importante de contaminación de alimentos, pero sólo en los últimos años se han estudiado en mayor profundidad. Con estos estudios se pretenden identificar rutas y factores de riesgo asociados a la contaminación cruzada utilizando para ello un enfoque cuantitativo basado en modelos (den Aantrekker y col., 2003; Reij y col. 2004). Los escasos modelos que se encuentran en la bibliografía se basan en la aplicación de tasas de transferencia (TR), en porcentajes que describen la proporción de bacterias que pasan de

una superficie contaminada a otra debido al contacto entre ellas (Zhao y col., 2000):

$$TR\ (\%) = \frac{ufc\ en\ la\ superficie\ donadora}{ufc\ en\ la\ superficie\ receptora} X\ 100$$

Modelos secundarios

Los modelos secundarios son ecuaciones matemáticas más complejas, ya que relacionan los parámetros de cinéticos de los modelos primarios con factores ambientales tanto intrínsecos como extrínsecos, tales como: pH, temperatura, actividad de agua, etc. Entre los modelos secundarios más utilizados, encontramos (Ross y Dalgaard, 2004):

1. Modelos polinomiales
2. Modelos de raíz cuadrada
3. Modelos gamma
4. Modelos de redes neuronales artificiales

Los modelos de mayor utilización son los modelos polinomiales o también conocidos como: "modelos de superficie de respuesta (MSR)" (Baranyi y col., 1996). Estos presentan interesantes ventajas que lo hacen preferibles:

- ✓ Presentan un buen ajuste con datos obtenidos experimentalmente.
- ✓ Son de fácil desarrollo matemático.

Sin embargo, estos modelos poseen importantes limitaciones que deben considerarse antes de su utilización. En primer lugar no permiten extrapolación y deben aplicarse dentro del dominio de desarrollo del modelo (región de interpolación). A diferencia de lo que ocurre en otros modelos como el Modelo gamma, los MRS no pueden ser fácilmente extendidos a nuevos factores o rangos y requerirían un nuevo tratamiento de datos. Finalmente, estos modelos pueden presentar problemas de sobreajuste además de no permitir una interpretación de sus términos dado su carácter puramente empírico (Baranyi y col., 1996).

El modelo de raíz cuadrada se presenta como una alternativa a los MSR, en este tipo de modelos se encuentran los tipo Ratkowsky o Bêleradek que como base matemática tienen la siguiente función (Ratkowsky y col., 1982; McMeekin y col., 1993; Bernaerts y col., 2004):

$$\sqrt{\mu_{max}} = b \cdot (T - T_{min})$$

donde μ_{max} es la tasa máxima específica de crecimiento (h^{-1}), b es una constante y T es la temperatura y T_{min} es la temperatura mínima teórica a partir de la cual se detecta crecimiento.

Esta función puede extenderse para comprender el rango biocinético completo, incluyendo como parámetro la temperatura máxima teórica (T_{max}), valor por encima del cual no existe crecimiento (Ratkowsky y col., 1983)

$$\sqrt{\mu_{max}} = b\ (T - T_{min})\ +\ [1 - \exp(c \cdot (T - T_{max}))]$$

Esta ecualización también ha sido ampliada para incluir otros factores ambientales tales como a_w, pH, CO_2, etc. (Ross et al. 2003)

En el caso de los modelos gamma, cabe destacar que fue Zwietering (1992), quien introdujo este concepto fundamentándolo en dos principios generales:

1. Los factores ambientales afectan el crecimiento bacteriano de manera independiente; y por tanto su efecto total es igual a la multiplicación de los

efectos individuales de cada factor.

2. La tasa máxima de crecimiento (μ_{max}) es igual a una fracción de la tasa máxima de crecimiento óptima (μ_{opt}) la cual resulta de multiplicar el efecto inhibitorio de cada factor ambiental por la tasa máxima óptima de crecimiento.

El efecto inhibitorio viene dado para cada factor por una función llamada Gama (γ). El termino gamma es adimensional y se encuentra entre 0 y 1, donde el valor 1 corresponde a ausencia de inhibición. Cuando los valores de los factores son óptimos para el crecimiento, las funciones γ toman un valor de 1, siendo por tanto la tasa de crecimiento estimada igual a la tasa de crecimiento óptima. Este modelo secundario es de gran aplicabilidad en la ECRM y permite ser extendido con nuevos factores ambientales (Wijtzes, 2001, Zwietering, 1996).

$$\mu_{\max} = \mu_{opt} \quad \gamma(T)\,\gamma(pH)\,\gamma(a_w)$$

Los modelos de redes neuronales artificiales presentan un excelente ajuste y sofisticación. La arquitectura de una red neuronal consiste en la organización de neuronas en red formando diferentes capas o agregaciones como se muestra en los ejemplos de la Figura 6. El desarrollo de modelos de redes neuronales presenta una elevada complejidad. Esta hace difícil su aplicación en hojas de cálculo, de las ecuaciones o funciones y sus parámetros (García-Gimeno y col., 2002, Schepers y col. 2000; Valero y col. 2007; Oscar 2009).

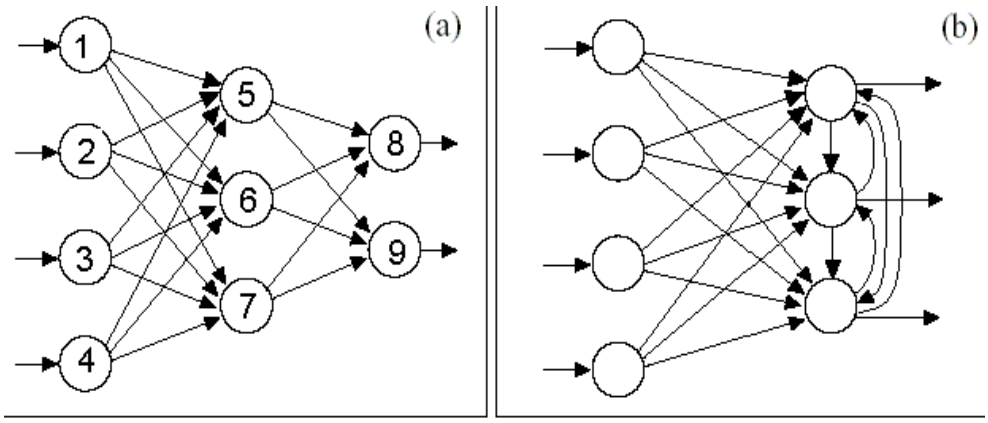


Figure 6. Ejemplos de modelos de redes neuronales

Los modelos secundarios más frecuentes son aplicados a la tasa de crecimiento máximo. También existen modelos secundarios aplicados al tiempo de latencia pero son más inexactos e imprecisos debido a que el tiempo de adaptación se encuentra relacionado con las condiciones pre-cultivo (historia celular), mostrando estos una gran variabilidad (Swinnen y col. 2004, Baty y col. 2004). Por ello, en el caso del lag, los modelos matemáticos que mejor se adaptan son los modelos estocásticos. Los modelos estocásticos están basados en el uso de distribución de probabilidad, describiendo la frecuencia de aparición de los distintos valores de tiempo de latencia (Robinson, 1998; Swinnen y col. 2004; Olofsson y col. 2011).

Los modelos secundarios basados en la densidad máxima son otro conjunto de modelos secundarios no muy desarrollados especialmente para microorganismos patógenos dado que hay otros parámetros de mayor importancia en el riesgo como son el tiempo de

latencia y tasa de crecimiento. Sin embargo, en el caso de los microorganismos alterantes estos modelos si cobran mayor importancia dado que su capacidad de alteración se encuentra muy relacionada con los niveles máximos de población; si bien, a pesar de su aplicabilidad, existen pocos modelos para este tipo de microorganismos (Zurera-Cosano y col. 2006).

Los modelos primarios tradicionalmente se han desarrollado en condiciones estáticas para los factores ambientales en estudio. No obstante, en la práctica, difícilmente, las condiciones ambientales se mantienen constantes en el tiempo. De ahí que la utilización de modelos que consideren el comportamiento microbiano en condiciones dinámicas sea de gran importancia para obtener predicciones más exactas. A pesar de su relevancia son aún pocos los modelos desarrollados en condiciones dinámicas (Bernaerts y col., 2004). Hay diferentes modelos primarios que pueden ser utilizados para reflejar condiciones cambiantes (Baranyi y col. 1993; Hills y Mackey 1995; McKellar y Delaquis, 2011). El modelo de Baranyi y Roberts (Baranyi y Roberts, 1994) es quizás el modelo que más se ha aplicado a condiciones dinámicas ya que permite ser adaptado desde su función diferencial para reflejar la variación de los parámetros cinéticos con respecto al tiempo. Este se ha utilizado tanto para predecir el crecimiento como muerte microbiana en diferentes alimentos como han demostrado Pin y col. (2011) o Psomas y col. (2011).

Modelos terciarios

Los modelos terciarios son aplicaciones informáticas que integran los modelos primarios y secundarios que permiten a los usuarios finales aplicar los modelos predictivos en diferentes contextos para obtener predicciones del comportamiento microbiano en los alimentos (crecimiento o muerte).

MicroHibro ha sido creado en 2012 por el grupo de investigación HIBRO de la Universidad de Córdoba, nueva herramienta de predicción on-line para productos vegetales y cárnicos. Es una base de datos de modelos de crecimiento y supervivencia de los microorganismos que permite hacer predicciones en condiciones ambientales específicas. Asimismo incorpora funcionalidades para la inclusión de modelos por usuarios expertos en microbiología predictiva. También incorpora un módulo de validación para evaluar los modelos disponibles con sus propios datos. En un segundo módulo la aplicación incorpora un enfoque estocástico destinado a la evaluación de riesgo.

Pathogen Modeling Program “PMP”, fue creado por USDA en Eastern Regional Research Center, Microbial Food Safety Research Unit; y proporciona información de estimaciones de patógenos en procesos de inactivación y crecimiento en función de diferentes factores ambientales.

Combase fue desarrollado por el Instituto de Investigación Alimentaria del Reino Unido (IFR, UK) y está integrado al Departamento de Agricultura de los Estados Unidos y Centro de Seguridad Alimentaria de la Universidad de Tasmania y Australia (Baranyi y Tamplin 2004). Es una base de datos que contiene una recopilación de modelos predictivos relevantes en una amplia variedad de ambientes (caldos de cultivo y alimento) lo que proporciona informacion de crecimiento, inactivación y supervivencia microbiana. Está compuesto por diferentes herramientas:

- ComBase Browser: Es la base de datos de crecimiento e inactivación, recopiladas en los centros de investigación y de las Publicaciones científicas.
- ComBase Predictor: predece el comportamiento de microorganismos patógenos y

alterantes frente a los factores ambientales.

- Perfringens Predictor: Es una aplicación especialmente que predece el crecimiento de *Cl. perfringens* durante la refrigeración de carne sometida a proceso de cocción.

Tambien existen otros modelos terciarios de cierta relevancia en diferentes ámbitos: Seafood Spoilage And Safety Predictor (SSSP), Microbial Responses Viewer (MRV) (Koseki, 2009), SYM´Previus (Leporq y col.2005).

Elaboración de modelos predictivos

El desarrollo de modelos predictivos comprende multitud de métodos y enfoques según microorganismo, matriz, y fines del modelo. Existen numerosas revisiones científicas sobre cómo abordar el desarrollo de modelos, para una mejor comprensión de la metodología recomendamos su lectura detallada (Devlieghere y 2000; Rash, 2004; McMeekin et al. 1993). No obstante, a continuación presentamos un pequeño resumen sobre los elementos y fases principales para el desarrollo de modelos predictivos. Los modelos predictivos pueden construirse con datos ya existentes o de nueva generación, en el propio alimento o en caldo artificial. En caldo de cultivo artificial o extracto se pueden generar mayor número de experimentos, además de posibilitar un mejor control de las condiciones y un menor uso de recursos tanto humanos, materiales y de tiempo. En contraposición estos modelos se van a producir predicciones que pueden ser diferentes a las observadas en condiciones reales, por tanto requerirán una validación para su aplicación. Los modelos generados en el alimento proporcionaran estimaciones más exactas por el hecho de haber sido desarrollados en matrices reales. A pesar de ello, estos modelos suelen estar limitados en el dominio de aplicación dado que desarrollan en un rango estrecho de condiciones ya que requieren un mayor gasto en recursos, una inoculación compleja y generan una elevada variabilidad de los resultados.

Los datos necesarios para la construcción de los modelos pueden ser generados a través del recuento de placa, observaciones al microscopio, impedancia o densidad óptica (este método por turbidez es el más utilizado). La obtención de los modelos matemáticos se realizan mediante métodos de ajuste a través de la regresión; lineal, no lineal y redes neuronales. La validación es una fase necesaria previa a la aplicación de los modelos desarrollados que permite evaluar si el modelo desarrollado es adecuado para su aplicación en los diferentes contextos alimentarios. Existen dos tipos de validaciones, interna y externa. La validación interna es aquella que evalúa la capacidad del modelo para reproducir observaciones generadas en el mismo experimento. Para ello es necesario generar dos conjuntos de datos, el primer conjunto se destina para generar los modelos y el segundo conjunto de datos es utilizado para realizar la validación. En el caso de la validación externa se realiza con las observaciones obtenidas en el alimento con el fin de evaluar la exactitud de la predicción del modelo.

Aplicaciones de los modelos predictivos

El uso de modelos de microbiología predictiva es muy diverso pero en general podemos resumirlo según el planteamiento de Membré y Lambert (2008), en tres aplicaciones: asistencia en alertas alimentarias, innovación de alimentos y/o procesos, toma de decisiones. En el caso de asistencia en alertas alimentarias nos referimos a la estimación que se puede realizar en cuanto a las consecuencias y repercusiones sobre la seguridad del consumidor, si se presentara algún problema durante la cadena de producción y en especial durante la comercialización y almacenamiento de los alimentos. Asimismo, en el momento de tomar decisiones permite ayudarla declinarse por una de las opciones planteadas gracias al conocimiento de los posibles panoramas bajo esas condiciones;

esta aplicación es de mucha ayuda a la hora de llevar a cabo medidas de seguridad alimentaria, como establecimiento de los PCC, en los sistemas APPCC, en cambios en el diseño del proceso. por último, y no menos atractivo para la industria se encuentra la innovación de alimentos, ya que la microbiología permite predecir y esto a su vez facilita la evaluación de los productos en cuanto a un posible crecimiento bacteriano, a el tiempo necesario para que superen concentraciones de riesgo y la velocidad de los microorganismos para colonizar un producto, toda esta información es clave a la hora de diseñar, desarrollar, reformular, optimizar los procesos de producción y a su vez los productos. Otra aplicación de la microbiología Predictiva es que forma parte de una de las piezas claves en la ECRM, Evaluación de la Exposición (ver siguiente apartado).

Evaluación Cuantitativa del Riesgo Microbiológico (ECRM)

El concepto de Evaluación de Riesgo Microbiológico ERM forma parte de un concepto o temática más amplio, esto es Análisis del Riesgo (FAO/WHO, 1995). El Reglamento (CE) Nº 178/2002 del parlamento europeo y del consejo de 28 de enero del 2002 afirma que: "Análisis del riesgo es el proceso formado por tres elementos interrelacionados: Evaluación del riesgo, Gestión del riesgo y comunicación del riesgo."

El análisis de riesgo se fundamente en bases científicas que justifican y sustentan las decisiones y acciones a tomar, en cuanto a un determinado peligro o riesgo alimentario (FAO/WHO, 1995). El objetivo fundamental del Análisis del Riesgo es lograr un nivel elevado de protección de la salud de los consumidores. La evaluación del riesgo y la gestión del riesgo deben estar integradas con la comunicación del riesgo como así queda reflejado en la Figura 7.

Figura 7. Representación de la interacción entre los diferentes componentes del Análisis del Riesgo (FAO/WHO, 1995).

Las directrices generales que se deben seguir para realizar una Evaluación del Riesgo Microbiológico (ERM) vienen reflejadas en el documento de la Comisión del Codex Alimentarius CAC (1999). En este documento se definen las fases de las que debe constar un proceso de ERM y que definimos a continuación

1. *Identificación del Peligro (IP).* Identificación de agentes biológicos, químicos o físicos capaces de causar un potencial efecto adverso sobre la salud y que pueden estar presentes un determinado alimento o grupo de alimentos.

2. *Caracterización del Peligro (CP)*. La evaluación cuantitativa o cualitativa de la naturaleza de los efectos nocivos para la salud asociados con el peligro en cuestión. Para los fines de la evaluación de riesgos microbiológicos, son objeto de interés los microorganismos y/o sus toxinas.

3. *Evaluación de la Exposición (EE)*. Evaluación cuantitativa y/o cualitativa del grado de ingesta de un microorganismos y/o sus toxinas a través de alimentos o de otras fuentes si son relevantes.

4. *Caracterización del Riesgo (CR)*. El proceso de determinación de la estimación cualitativa y/o cuantitativa, incluidas las incertidumbres que conlleva, de la probabilidad de aparición y gravedad de efectos adversos conocidos o potenciales para la salud de una población dada, sobre la base de la identificación del peligro, la caracterización del mismo y la evaluación de la exposición.

En el desarrollo de un estudio de ECRM se deben comenzar por definir el objeto de la misma (Morgan y Henrion 1990) y establecer las estrategias a llevar a cabo a lo largo del estudio y en especial sobre las poblacionales de riesgo (que pudieran requerir un tratamiento específico en la ECRM). En cuanto a otros aspectos, también se puede indicar el tipo de análisis de los datos, tratamiento de la incertidumbre, escenarios a modelar, etc. La ERM se fundamenta en la aplicación de modelos, esencialmente en las etapas de Evaluación de la Exposición (EE) y Caracterización del Riesgo (CR) (Lammerding y Paoli, 1997).

Objetivo de Seguridad Alimentaria (FSO)

En 1998 la International Commission of Microbial Specifications in Foods (ICMSF) propone el uso del concepto “Food Safety Objetive” (FSO) como un puente de unión entre objetivos y políticas de Salud Pública y la gestión del riesgo a lo largo de la cadena alimentaria (ICMSF, 1997 y 1998). Este concepto se define más tarde “como la frecuencia y/o concentración máxima de un peligro microbiano en un alimentos en el momento de consumo que proporciona un Nivel adecuado de protección (ALOP)” (ICMSF, 2002). Por tanto, el FSO se propone como elemento clave en el desarrollo de los sistemas de gestión de riesgos microbiológicos, proporcionando una base cuantitativa para el desarrollo de medidas de control, y diseño e implantación de sistemas de autocontrol (Zwietering, 2005).

Siguiendo los pasos de la ICMSF, la FAO ha desarrollado su propio marco de gestión basado en el FSO (FAO/WHO, 2004). Según esta última, el FSO puede ser establecido bien en base a datos epidemiológicos que describen el estatus de salud pública actual en cuanto a un peligro o bien mediante la aplicación de una curva de caracterización del riesgo. En este último caso, existe una base cuantitativa para relacionar el FSO y el ALOP, base indiscutiblemente vinculada a una ERM de indole cuantitativa, donde variables tanto de “salida” o “entrada” pueden relacionarse con el FSO.

Para facilitar su aplicación por los operadores alimentarios, la ICMSF (2002) propone la siguiente inecuación que considera de forma resumida el efecto de los distintos procesos y subprocesos (crecimiento, recontaminación y reducción) y la sumatoria de todo ellos permiten o no alcanzar un FSO:

$$H_0 + \sum I + \sum R \leq FSO$$

Ho es la población inicial de microorganismos, I es un factor de incremento y R es un factor de reducción. Todos los términos de la inecuación son expresados en log10

La presente inecuación es una valiosa herramienta para la validación de medidas preventivas o medidas de control, proporcionando una base cuantitativa para ello (Zwietering y col., 2010). Recordamos que Validación, según el Codex Alimentarius, "es el proceso a través del cual se garantiza que una medida de control definida es capaz de mantener bajo control un peligro concreto en un alimento específico" (CAC, 2001). Los modelos predictivos son herramientas, implícitas en la inecuación, que pueden aplicarse también a tal efecto, sin menos cabo de la utilización de información científica, pruebas experimentales, datos históricos etc. como se recoge en diversos documentos (CAC, 2001). Este sistema puede aplicarse a lo largo de las diferentes etapas de la cadena alimentaria mediante la utilización de objetivos o estándares de seguridad alimentaria específicos (FAO/WHO, 2004):

Objetivo de Rendimiento (OR): "la máxima frecuencia y/o concentración de un peligro en un alimento, en un fase específica de la cadena alimentaria antes del momento de consumo, que proporcione o contribuya al logro de un FSO o un ALOP, según corresponda".

Criterio de Rendimiento (CR): "el efecto que debe ser logrado en la frecuencia y/o concentración de un peligro en un alimento por medio de la aplicación de una o más medidas de control para lograr o contribuir a lograr un OR o un FSO."

Cuantificación y modelización de procesos microbiológicos en la Industria de Vegetales IV Gama: Un enfoque para la gestión

La seguridad alimentaria en el sector de los productos IV Gama esta marcada por dos aspectos importante. Estos hacen referencia, por un lado a que los tratamientos de higienización aplicados en la industria no son completamente efectivos eliminando la carga microbiana presente, y por otro lado, al hecho de que los productos son consumidos crudos (Francis y col., 1999). Por ello, la inocuidad microbiológica de estos productos depende en gran medida de la aplicación de la "teoría de obstáculos", donde diferentes factores y tecnologías de conservación son combinadas con el fin de reducir la contaminación y/o inhibir el posible crecimiento microbiano a lo largo de toda la cadena alimentaria (Lee, 2004). Entre los factores clave en el control de los patógenos en vegetales mínimamente procesados se señalan, como los más importantes, los procesos de higienización y la temperatura de almacenamiento y distribución, puesto que se ha demostrado que los microorganismos patógenos pueden sobrevivir a una exposición al cloro y posteriormente crecer si las condiciones son idóneas para ello (Ana y col., 2012; Delaquis y col., 2007; Franz y col., 2010; Legnani y Leoni, 2004; Tromp y col., 2010). En este sentido, una evaluación de riesgos de *E. coli* O157:H7 en vegetales de hoja identificó la temperatura y tiempos de almacenamiento junto a la contaminación durante la etapa de lavado como los factores de riesgo más importantes (Danyluk y Schaffner, 2011). El tratamiento de lavado con agua clorada con niveles de 20-200 ppm de cloro libre, el rango de aplicación por la Industria, produce reducciones de entre 1 y 3 unidades logarítmicas decimales (Aruscavage y col., 2006), lo que indicaría la posibilidad, en algunos casos, de supervivencia y/o recuperación de los microorganismos a este proceso (Kolling y Matthews, 2007; Zhao y col., 2001). Por

otro lado, Carrasco y col. (2007) encontraron que los frigoríficos domésticos en España podrían permitir el crecimiento de patógenos tales como *L. monocytogenes*, *E. coli* O157:H7 y *Salmonella* spp. en vegetales de hoja teniendo en cuenta el comportamiento y hábitos de los consumidores con respecto a los productos IV Gama.

Con el fín de establecer medidas preventivas que garanticen la seguridad de estos productos, es de vital importancia la cuantificación del efecto de estos procesos alimentarios sobre la supervivencia y crecimiento de los patógenos en los vegetales de IV Gama. La aplicación de modelos predictivos en un contexto de evaluación de la exposición o riesgo puede resultar en la identificación de etapas críticas, factores de riesgo o parámetros de proceso. Tromp y col. (2010) señalaron la necesidad de contar con más datos cuantitativos para abordar estudios de evaluación de riesgos sobre las cadenas de distribución. Por otro lado, Danyluk y Schaffner, (2011), en relación con el riesgo de *E. coli* O157:H7 en vegetales de hoja, evidenció que se requiere más información sobre el tiempo de latencia de los microorganismos, su crecimiento, y el impacto de la contaminación cruzada durante la etapa de lavado.

Cuantificación y Modelos de desinfección en vegetales

La mayoría de los modelos de desinfección para patógenos han sido desarrollados para suspensiones acuosas de bacterias, con una aplicación principal en el tratamiento de agua de bebida. En estos casos, los modelos pueden ser desarrollados teniendo en cuenta un enfoque "vitalista" o "mecanicista" en el diseño de la función matemática (Lambert y Johnston, 2000). Los agentes desinfectantes se reducen durante el proceso de desinfección debido a la interacción con otras sustancias contenidas en el medio, como ocurre con el hipoclorito al contactar con materia orgánica (Winward, Avery, Stephenson, & Jefferson, 2008) o se disipan dada su volatilidad. Por ello, existen modelos, como los derivados de la familia de Weibull que consideran el carácter no estático de la concentración del agente a lo largo del proceso aplicando para este fin ecuaciones diferenciales (Corradini y Peleg, 2007).

$$\frac{dS(t)}{dt} = -b[C(t)] \cdot n \cdot \left[\frac{dS(t)}{-b[C(t)]} \right]^{\frac{n-1}{n}}$$

donde S es la reducción logarítmica de células, $b[C(t)]$ es un coeficiente dependiente de la concentración del agente desinfectante que varía con el tiempo (t) , *n* es otro coeficiente asumido constante para los diferentes valores de C(*t*).

En el caso de productos IV Gama, el problema radica en que el proceso de desinfección debe ser efectivo tanto para el agua de lavado como para el alimento tratado. En el primer caso, es crucial para reducir la contaminación cruzada durante el proceso de lavado (Gil y col., 2009), y en el segundo, para minimizar el riesgo de presencia de patógenos en el alimento y mejorar su calidad y vida comercial. Por tanto, parece claro que dos tipos de modelos deben aplicarse para tener una completa representación del proceso de desinfección, uno aplicado para el agua de lavado y otro para la desinfección en superficie del vegetal. Para el primer tipo de modelos existen datos abundantes y modelos aplicados a tratamiento de agua que podrían ser equivalentes o adecuados para este caso. En el párrafo anterior hemos dados una pequeña pincelada sobre los mismos, si bien, una mayor detalle sería necesario dado que en si mismo es un área científica específica. Los procesos de desinfección han sido estudiados de manera exhaustiva para diferentes tipologías de tratamientos, tradicionales y alternativos, como se ha comentado anteriormente.

El proceso más frecuentemente estudiado es la utilización de hipoclorito en el agua de lavado, siendo este el tratamiento más utilizado por la industria alimentaria en España y otros países (EEUU). Existen multitud de estudios que examinan y cuantifican la eficacia del cloro en la reducción de la carga microbiana en el alimento, tanto total, indicadora de higiene, como patógena. Estos evidencian un comportamiento variable, probablemente debido a la multitud de factores implicados en su mecanismo de acción y a la dificultad de mantener todos ellos bajo control, especialmente cuando los experimentos son desarrollados a gran escala (Behrsing y col., 2000). Este hecho ha llevado a que, con contadas excepciones (Pirovani y col., 2004), este proceso haya podido describirse mediante la utilización de modelos matemáticos deterministas, es decir, funciones matemáticas que relacionen los factores implicados y la reducción del patógeno en el alimento a lo largo del tiempo. Por ello, se ha propuesto de una manera más generalizada el uso de modelos estocásticos, a través de distribuciones de probabilidad que permitan tener en cuenta la variabilidad observada y la frecuencia o probabilidad de los distintos niveles de eficacia, o reducción logarítmica (Carrasco, y col., 2010; Danyluk y Schaffner, 2011; Franz y col., 2010; Tromp y col., 2010). Es evidente, que los procesos de desinfección pueden ser clave en la reducción del riesgo, especialmente si los parámetros de procesos se controlan y se mantienen a los valores óptimos. No obstante, es igualmente patente, que una reducción total no es factible hoy en día (Beuchat y col., 2001; Lang y col., 2004), por lo que es de especialmente relevancia las fases posteriores tanto por los procesos de contaminación cruzada como por el potencial crecimiento posterior que podría ser clave en la aparición de brotes alimentarios. Esto toma mayor relevancia cuando se tiene en cuenta que la canales de distribución de estos productos se realizan cada vez a mayor escala, aumentado la probabilidad de aparición de fallos en la cadena de frío, a la vez que se incrementa la posibilidad de crecimiento de los microorganismos que pueden haber sobrevividos a los tratamientos de higienización.

Cuantificación y Modelos predictivos de crecimiento de microorganismos patógenos en vegetales de hoja

Aunque existen multitud de trabajos que han estudiado el crecimiento potencial de microorganismos patógenos en vegetales de hoja (Amanatidou y col., 1999; Francis & Beirne, 2001; Ongeng y col., 2007; Sant'Ana y col., 2012; Valero y col., 2006), son pocos los que han desarrollado modelos predictivos específicos para estos productos (Crépet y col., 2009;Koseki y Isobe, 2005; McKellar y Delaquis, 2011; Sant'Ana y col., 2012). Tanto modelos como datos indicarían que los microorganismos, *L. monocytogenes, Salmonella* spp. y *E. coli* O157:H7 podrían crecer a temperaturas de refrigeración de 7-8 C, incluso bajo condiciones de envasado con atmósfera modificada (McKellar y Delaquis, 2011; Sant'Ana, Franco y col., 2012). Se ha documentado que *E. coli* O157:H7 podría crecer entorno a 2 logaritmos en 3 días a una temperatura de 12 C bajo atmósfera modificada (Luo y col., 2010). Por otro lado, temperaturas inferiores no permitirían el crecimiento del patógeno o llevarían a su inactivación (Oliveira y col., 2010). Los modelos desarrollados han sido modelos de crecimiento secundarios que permiten predecir la tasa de crecimiento, tiempo de latencia y densidad máxima en vegetales en función de la temperatura de almacenamiento. La mayoría de estos modelos predictivos han sido desarrollados con microorganismos inoculados en el alimento posteriormente al tratamiento y/o envasado, o simplemente sobre productos no tratados, y pocos han tenido en cuenta el efecto del tratamiento de desinfección sobre el crecimiento posterior del microorganismo (Koseki y Itoh, 2001; McKellar y Delaquis, 2011). El estudio realizado por Koseki y Itoh (2001) registró un mayor crecimiento de

E. coli O157:H7 en lechuga y coliflor cuando estas fueron sometidas a un tratamiento con agua electrolizada en comparación a los productos no tratados. Los estudios consultados indican que aún no está claro si el cloro reduce la capacidad de crecimiento de *E. coli* O157:H7 o si bien, se ve aumentada a través de una reducción de la microflora competidora (Delaquis y col., 2002). En todo caso, el empleo de modelos no ajustados o validados en las condiciones comerciales de los productos de IV Gama podrían derivar en importantes desviaciones en las predicciones, y en algunos casos, estas podrían ser no seguras. Un mayor estudio del efecto de los proceso de higienización sobre el posterior crecimiento del patógeno y el desarrollo de los modelos predictivos correspondiente permitiría valorar con mayor exactitud el riesgo de crecimiento de los diferentes patógenos a lo largo de la cadena de distribución de los vegetales de IV Gama.

Cuantificación y Modelos de transferencia y/o contaminación cruzada en productos IV Gama.

Los fenómenos de contaminación cruzada son de gran relevancia en el sector de los productos IV debido a que en el proceso de lavado, el agua puede actuar como vehículo de transmisión entre productos contaminados y no contaminados. Para reducir el riesgo de contaminación cruzada, la industria aplica durante el lavado compuestos desinfectantes o higienizantes, siendo el cloro el más ampliamente utilizado (López-Gálvez y col., 2009; Tomás-Callejas y col., 2012). A este respecto se ha sugerido que la contaminación cruzada podría ser el factor de riesgo determinante en la aparición de brotes alimentarios asociados a productos vegetales de IV Gama. Como principal argumento se indica que la contaminación cruzada produce una homogenización y/o redistribución de la contaminación de patógenos en las unidades de lote durante el lavado (Wachtel y Charkowski, 2002), lo que podría ocurrir cuando se reduce la eficacia del producto higienizante debido a una mayor presencia de materia orgánica o a un control deficiente del proceso (Olaimat y Holley, 2012). Sin embargo, la contaminación cruzada por si sola no es suficiente, y para la aparición de brotes alimentarios se requiere además la ocurrencia de temperaturas de abuso, o largos periodos de tiempo con temperaturas que permitan el crecimiento del patógeno a niveles de riesgo (Rosset y col., 2004). También en línea con estas afirmaciones, el estudio de evaluación de riesgos llevado a cabo por Danyluk y Schaffner (2011) estimó que cuando el producto estuvo contaminado con un 0.1% del patógeno *E. coli* O157:H7 el 99 % de los casos originados se derivaron de productos contaminados durante la etapa de lavado. Estudios recientes realizados en plata piloto demuestran la capacidad de E. coli O157:H7 para transferirse durante las diferentes etapas en el procesado de vegetales de hoja cortados, contaminando superficies, agua y producto final (Buchholz y col., 2012a, 2012b). En el estudio de Buchholz y col. (2012b) se demostró que un nivel de inoculo inicial de 2 log ufc/g de *E. coli* O157:H7 en la lechuga inoculada resultó en la contaminación al final de proceso de 21.2 de los 78 kg de lechuga inicialmente no inoculada, donde los niveles oscilaron entre 2 y 3 log ufc/100 g. Este resultado pone de relevancia la capacidad del microorganismo para transferirse desde las superficies, equipos (cortadora, cinta transportadora, centrifugadora, etc.) y agua de lavado a lo largo del proceso, extendiendo la contaminación a un mayor número de unidades de producto finales.

Los procesos de cortado o manipulación tanto en la etapa industrial y en etapas posteriores durante su distribución, venta y consumo podrían ser causas de recontaminación del producto como han evidenciado los diversos trabajos para tanto *E. coli* O157:H7 como para Salmonella spp. (Ravishankar y col., 2010; Gorman y col.,

2002; Wachtel y Charkowski, 2002). En uno de estos trabajos, se demostró que *E. coli* O157:H7 podría transferirse desde carne cruda contaminada a hojas de lechuga a través de la tabla de cortado, documentándose que el 45 % de las hojas (n=25) en contacto con la tabla de corte fueron contaminadas con el patógeno (Wachtel y col., 2003).

A pesar de existir datos cuantitativos sobre la transferencia o contaminación cruzada de patógenos en los procesos de lavado de productos IV Gama, no se han incorporado en modelos predictivos (Pérez-Rodríguez y col., 2008). Su desarrollo podría ser interés como herramienta valiosas en los procesos de toma de decisiones, sistemas cuantitativos de gestión del riesgo, y evaluación cuantitativa del riesgo microbiano, además de su aplicación para el establecimiento y selección de métricas de riesgo, FSO, PO, PC, parámetros de proceso u otros criterios de seguridad alimentaria.

Aplicación de modelos predictivos para la gestión cuantitativa de la seguridad de los alimentos IV Gama basada en el FSO y en el empleo de la inecuación de la ICMSF (2002)

Para la derivación de un FSO sobre una base cuantitativa, se requiere la realización de estudios exhaustivos de evaluación de riesgo que permiten relacionar a través de una curva de caracterización la dosis ingerida con un objetivo de salud pública , expresado en número de casos, o a través de otros índices, como los años de vida potencialmente perdidos (DALYs) (Havelaar y col. ,2008; Zwietering, 2005). Se han realizado estudios que han utilizado modelos probabilísticos con el fin de derivar un FSO, y otras métricas de riesgo, al igual que criterios microbiológicos cuando se ha requerido (Delignette-Muller y Cornu, 2008; Gkogka y col., 2013; Mejia y col., 2011). Este puede ser el caso de un estudio sobre *Clostridium perfringens* en carne lista para el consumo o parcialmente cocida, donde se ha desarrollado un modelo probabilístico de segundo orden que estima el riesgo asociado a este microorganismo y tipología de productos y a partir de esta base cuantitativa se derivan diferentes métricas de riesgos, medidas de control y criterios microbiológicos, utilizando para ello un enfoque de escenarios (Crouch y col., 2009). Para este tipo de estudios es crucial contar con modelos predictivos que permitan describir con precisión las diferentes etapas de la gran a la mesa y su relación con el riesgo final (Buchanan y Appel, 2010).

La inecuación propuesta por ICMSF (2002) es una simplificación y sistematización de la etapa de evaluación de la exposición en el esquema de evaluación de riesgos (CAC, 1999). Esta adaptación permite a los industriales y diferentes operadores de la cadena alimentaria cuantificar el efecto sobre los microorganismos de las diferentes etapas en la cadena alimentaria, a través de los tres términos mencionados anteriormente, esto es, incrementos, reducción y concentración inicial. La sumatoria de todos ellos debe resultar en un valor inferior o igual al FSO establecido, o si se aplica a un conjunto de etapas específicas, a un PO. De esta manera, los objetivos de salud pública y ALOP son trasladados de una manera eficiente al nivel operacional para así guiar la toma de decisiones, APPCC, y sistemas de gestión. Se ha propuesto por tanto su aplicación, para derivar o seleccionar objetivos y criterios de funcionamiento (Membré y col., 2007; Zwietering, 2005), la selección y validación de medidas de control (Schothorst y col., 2009), y parámetros de proceso y alimento (Gorris, 2005).

Un desarrollo de modelos y cuantificación de procesos alimentarios en el sector de vegetales, y especialmente, en el sector IV Gama sería crucial para guiar el desarrollo de estudios de evaluación de riesgos, y la puesta en funcionamiento de la inecuación de la ICMSF por industriales, como herramienta de gestión del riesgo. Como ha sido mencionado anteriormente, son múltiples los procesos que tienen lugar en la cadena

alimentaria de estos productos, si bien, un esquema tipo podría ser el representado en Figura 8, donde se establecen los procesos y parámetros de proceso críticos desde el punto de vista microbiológico. Para cada uno de ellos existen modelos predictivos que podrían aplicarse con el fin de conocer su efecto relativo sobre la capacidad del sistema de alcanzar un determinado FSO o PO, según el caso. A su vez, estos podrían utilizarse para determinar sobre qué parámetros de proceso deberíamos actuar para garantizar el cumplimiento de un FSO o PO.

Un ejemplo podría ser la aplicación de modelos de inactivación para describir las reducciones necesarias en la etapa de lavado, partiendo de una concentración inicial específica del peligro, sabiendo los incrementos producidos durante tanto el lavado como distribución del producto final. Si esto se aplica al caso de *E. coli* O157:H7 en vegetales de hoja, podríamos encontrarnos con el siguiente esquema:

- H_0= concentración inicial del patógeno en producto contaminado
- $\Sigma I = \Sigma I_{cc} + \Sigma I_{crecimiento}$
    - siendo ΣI_{cc} incrementos por contaminación cruzada durante la etapa de lavado y $\Sigma I_{crecimiento}$ los incrementos producidos por el crecimiento del patógeno a lo largo de la cadena alimentaria.
- $\Sigma R = \Sigma R_{cloro,}$ reducciones del patógeno sobre el vegetal debido a la aplicación de cloro en el agua de lavado
-

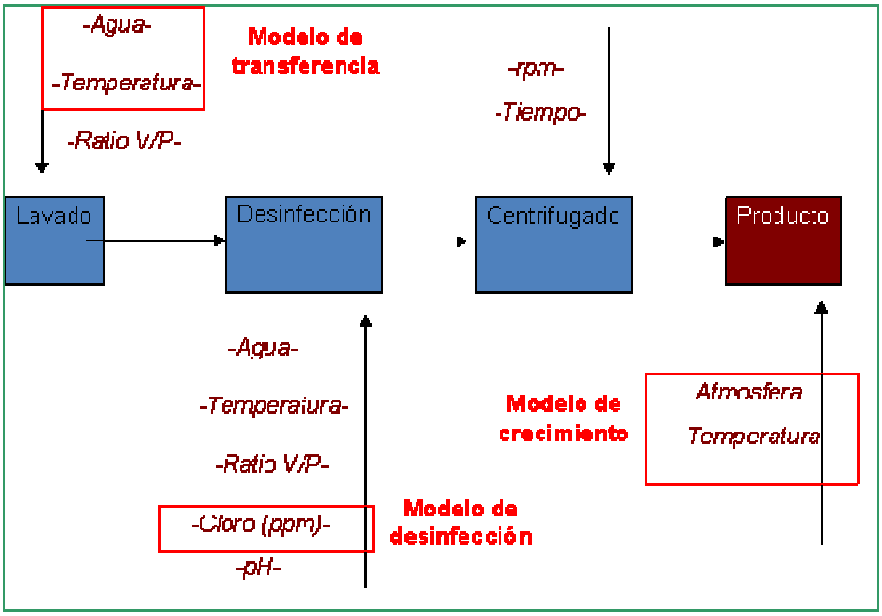


Figura 8. Un esquema de las operaciones y procesos básicos aplicados en la elaboración de productos IV Gama y la utilización de modelos predictivos para describir su efecto sobre microorganismos patógenos y derivar parámetros de proceso y otros medidas de control.

Para la concentración inicial H_0 es necesario realizar estudios de investigación y enumeración del patógeno; si bien, la prevalencia se encuentra a niveles tan bajos, que técnicamente es inviable el número de muestras necesarias para obtener valores

representativos de concentración. Por ello, en estos casos, el uso de diferentes escenarios de concentración es lo más adecuado.

En cuanto a los incrementos (ΣI), tanto por contaminación durante el lavado como por crecimiento, estos pueden extraerse desde estudios que proporcionan datos cuantitativos o bien mediante uso de modelos predictivos de transferencia y crecimiento, respectivamente. Tanto en el primer caso como en el segundo, los modelos predictivos permitirían relacionar parámetros de proceso como intensidad de lavado, concentración de cloro o temperatura de almacenamiento, o atmosfera modificada con los incrementos y en consecuencia con la capacidad de alcanzar el FSO o PO establecido. Si bien como se ha puesto de relieve anteriormente, los modelos son limitados en cuanto al tipo de patógeno, alimento y a las condiciones comerciales del producto, afectando ello a la exactitud de las estimaciones. Igualmente, ocurre en el caso de modelos de transferencia.

La reducción (ΣR) ocurre principalmente en la etapa de lavado con cloro. En este caso los modelos de inactivación permitirían relacionar el nivel de reducción con el nivel de cloro libre en el agua de lavado y tiempo de tratamiento. Nuevamente, no existen modelos secundarios específicos a las diferentes tecnologías de desinfección aplicadas. En todo caso, como un ejemplo hipotético con valores numéricos, que podrían estar derivados de modelos predictivos o extraídos de estudios cuantitativos, tendríamos como un hipotético FSO = -2 log cfu/g (1ufc/100g) ; H_0 = 1 log ufc/g; ΣI = 1 log ufc/g. Por tanto

$$-5 \geq -2\,(H_0) + 1(\Sigma I) + \Sigma R$$

$$\Sigma R = 4 \text{ log ufc/g}$$

Basado en estos cálculos se requiere una reducción en el producto de 4 logaritmos. Para alcanzar esta reducción deberíamos conocer la relación entre reducción logarítmica en el producto y los parámetros de proceso, en este caso, concentración de cloro libre, por ejemplo. Por otro lado, podría derivarse medidas de control combinadas, aplicando la "teoría de obstáculos" anteriormente mencionada. Así por ejemplo, si se reduce la sumatoria de incrementos a 0.5 debido a un control exhaustivo del lavado y de las temperaturas de almacenamiento a la vez que se reduce la concentración inicial a -3 log ufc/g, el número de reducciones necesarias sería 2.5 log ufc/g. Para derivar las temperaturas que requeriríamos para inhibir el crecimiento, tendríamos que aplicar modelos predictivos secundarios de crecimiento que contemplen la temperatura. En este caso, la temperatura sería un parámetro de proceso, y el valor obtenido un límite de control, concepto que enlaza con los sistemas APPCC. Los datos aquí aportados son un ejemplo, y han sido dados de manera determinista, no obstante, en un marco de evaluación de riesgos probabilístico, estas deberían ser descritas con distribuciones de probabilidad de esta manera se contemplarían todos los escenarios posibles, posibilitando un estimación más exacta y completa (Rieu, Duhem, Vindel, & Sanaa, 2007). A modo de resumen la Figura 9, se representa los modelos y factores a considerar en estudios cuantitativos basados en la inecuación de la ICMSF (2002) para el establecimiento y validación de medidas de control y establecimiento y selección de PO/PC en las distintas fases y procesos de la cadena de producción y distribución de los productos de IV Gama.

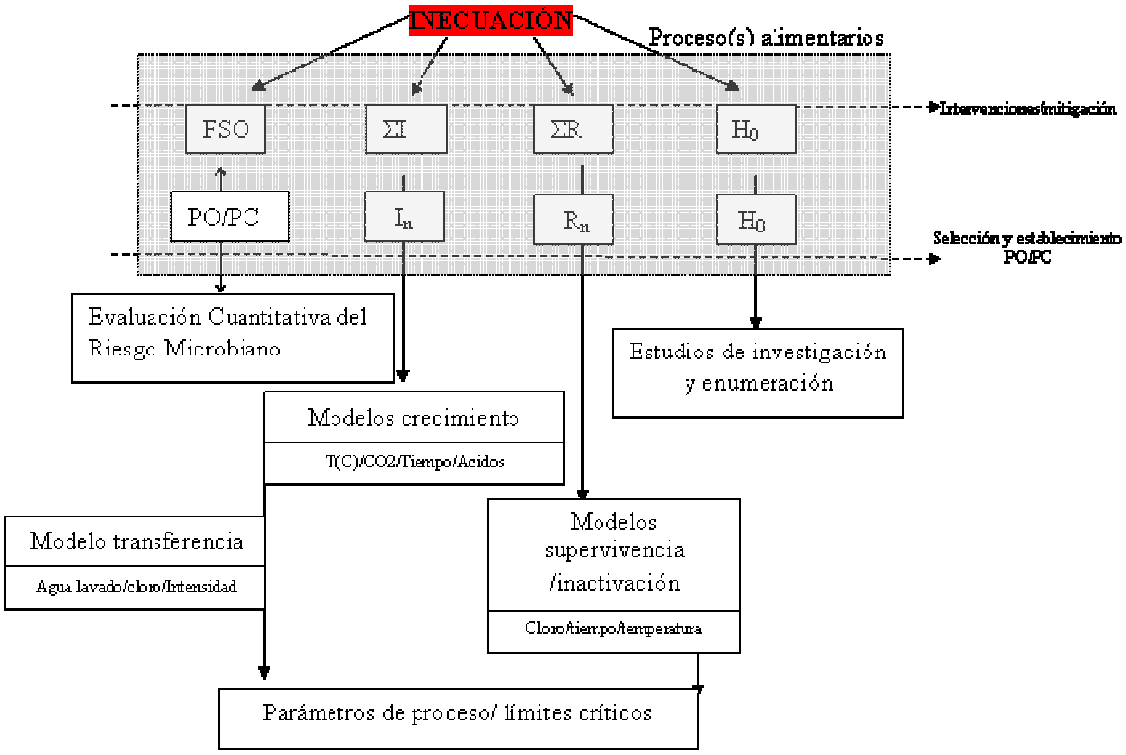


Figura 9. Un esquema resumen del desarrollo de un sistema cuantitativo de gestión de riesgo basado en los Objetivos de Seguridad Alimentaria y estructurado en la inecuación de la ICMSF (2002) y la aplicación de modelos predictivos.

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Objectives/Objetivos

The overall objective of this thesis is to study relevant and innovative aspects on microbial safety in Ready-to-Eat (RTE) vegetables, concerning specifically enteric pathogenic bacteria, and provide suitable predictive models to enable to simulate key food processes in the food chain of RTE vegetable.

Objective 1 To study and model the effect of traditional (hypochlorite) and innovative (electrolyzed water) decontamination and disinfection processes applied to leafy green vegetables, on pathogenic microorganisms. (*Chapters II, III and IV*)

Objective 2. To assess the effect of commercial and processing conditions, including disinfection treatments and packaging atmospheres, on the subsequent growth of pathogenic bacteria in RTE leafy vegetables. (*Chapters III and IV*)

Objective 3. To study and model survival of pathogenic bacteria in food environments concerning the RTE Vegetable Industry. This type of models is crucial to better understand and represent the effect of cross contamination events in the RTE vegetable Industry. (*Chapters I and V*)

Objective 4. To develop and provide suitable predictive models, particularly, growth, survival and transfer models for enteric pathogens in leafy green vegetables to fill in existing data gaps in studies of Quantitative Microbial Risk Assessment. (*All Chapters*)

Objective 5. To generate new quantitative knowledge and attendant mathematical models, in this field, in order to better support decision-making processes and to derive effective control measures in risk management systems of the RTE vegetable Industry. . (*All Chapters*)

El objetivo general de esta tesis es estudiar aspectos relevantes e innovadores sobre la seguridad microbiana de los vegetales IV Gama, específicamente en lo relativo a bacterias patógenas entéricas, y proporcionar modelos prediccitivos adecuados que permitan simular los procesos claves en la cadena alimentaria de los vegetales IV Gama.

Objetivo 1. Estudiar y modelizar el efecto de los procesos de descontaminación tradicionales (cloro) e innovadores (agua electrolizada) aplicados a vegetales IV Gama sobre microorganismos patógenos. . (*Capítulos II, III y IV*)

Objetivo 2. Evaluar el efecto de las condiciones comerciales y condiciones de procesado, incluyendo tratamientos de desinfección y atmósferas modificada en el envasado, sobre el crecimiento posterior de bacterias patógenas en vegetales IV Gama. (*Capítulos III y IV*)

Objetivo 3. Estudiar y modelizar la supervivencia de bacterias patógenas en ambientes alimentarios relacionados con la industria de vegetales IV Gama. Este tipo de modelos es crucial para entender y representar el efecto de la contaminación cruzada en la industria de vegetales IV Gama. (*Capítulos I y V*)

Objetivo 4. Desarrollar y proporcionar modelos predictivos, de crecimiento, supervivencia y transferencia, para patógenos en vegetales IV Gama que ayuden a resolver las actuales carencias en los estudios de la evaluación cuantitativa del riesgo microbiológico. (*Todos los Capítulos*)

Objetivo 5. Generar nuevos conocimientos cuantitativos así como modelos matemáticos que puedan aplicarse como herramienta en los procesos de toma de decisiones para la selección y establecimiento de medidas de control eficaces y optimización de los sistemas de gestión de riesgos de la industria de vegetales IV Gama. (*Todos los Capítulos*)

Results and Dicussion

Chapter I: "A mathematical risk model for *Escherichia coli* O157:H7 cross-contamination of lettuce during processing"

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Published in: Food Microbiology 28, 694-701, 2011

Abstract

A stochastic simulation modelling approach was taken to determine the extent of *Escherichia coli* O157:H7 contamination in fresh-cut bagged lettuce leaving the processing plant. A probabilistic model was constructed in Excel to account for *E. coli* O157:H7 cross contamination when contaminated lettuce enters the processing line. Simulation of the model was performed using @Risk Palisade© Software, providing an estimate of concentration and prevalence in the final bags of product. Three different scenarios, named S1, S2, and S3, were considered to represent the initial concentration on the contaminated batch entering the processing line which corresponded to 0.01, 1 and 100 cfu/g, respectively. The model was satisfactorily validated based on Standard Error of Prediction (SEP), which ranged from 0.00-35%. ANOVA analysis performed on simulated data revealed that the initial concentration in the contaminated batch (i.e., S1, S2, and S3) did not influence significantly (p=0.4) the *E. coli* O157:H7 levels in bags derived from cross contamination. In addition, significantly different (p< 0.001) prevalence was observed at the different levels simulated (S1; S2 and S3). At the lowest contamination level (0.01 cfu/g), bags were cross-contaminated sporadically, resulting in very low *E. coli* O157:H7 populations (mean: ≤2 cfu/bag) and prevalence levels (<1 %). In contrast, higher average prevalence levels were obtained for S2 and S3 corresponding to 3.05 and 13.39 %, respectively. Furthermore, the impact of different interventions on *E. coli* O157:H7 cross-contamination (e.g., pathogen testing, chlorination, irradiation, and cleaning and disinfection procedures) was evaluated. Model showed that the pathogen was able to survive and be present in the final bags in all simulated interventions scenarios although irradiation (0.5 KGy) was a more effective decontamination step in reducing prevalence than chlorination or pathogen testing under the same simulated conditions.

Introduction

*Escherichia co*li O157:H7 is a well-known pathogen capable of surviving under harsh environmental conditions and at refrigeration temperatures. Some pathogenic strains are also tolerant of low pHs (Duffy et al., 2000). An example of this is the cause of an outbreak associated with apple cider consumption in south-eastern Massachusetts where manure was used as a fertilizer in the orchard; *E. coli* survived in the apple juice for 20 days at pH values < 4, conditions previously considered sufficient to inhibit growth and survival of bacterial pathogens and non-toxigenic strains of *E. coli* (Besser et al., 1993). This unpredictable ability to survive at extreme conditions and the wide variability between strains makes it difficult to associate the incidence of the pathogen with a set of restricting conditions or parameters. Although it was originally associated with ground beef, the organism has more recently caused a series of outbreaks involving leafy salad vegetables including lettuce. Since 1993, at least 9 *E. coli* O157:H7 outbreaks have been traced to California-grown leafy greens – primarily lettuce and spinach (Cooley et

al., 2007). In 2006, a large nation-wide spinach outbreak occurred in US, that was linked to environmental *E. coli* O157:H7 in the field from wild animals or water (CDC, 2006). Other *E. coli* outbreaks may well have been linked to leafy salad vegetables but were not proven, e.g., an outbreak in 1995 involving leaf lettuce occurred in Montana affecting more than 70 people (Ackers et al., 1998), and another one in Minnesota in 2005 infected 12 people who ate bagged salads (Anonymous, 2005); in both outbreaks, the actual source of the pathogen remained without identification. Nevertheless, manure from farm animals was and is suspected as a leading vehicle of pathogen transmission. *E. coli* O157:H7 was isolated from sediments of an irrigation canal bordering a ranch that had been identified in three separate outbreaks in California (Doyle, 2000 a,b). The ability of the pathogen to survive in this type of environment has been proven in various works (Duffy, 2003, Johannessen et al., 2004). Most of these outbreaks involved packaged product that had wash and disinfection steps with hypochlorite, but were apparently insufficient to prevent *E. coli* O157:H7 from causing infections at the time of consumption. Although the exact *E. coli* contamination routes for these products remain unknown, an intermittent source of contamination within the processing environment may have occurred. In the thoroughly investigated 2006 spinach outbreak, the *E. coli* was found in the final bagged product of fresh-cut processed greens but originated at primary production (CDC, 2006). However, there is a lack of knowledge as to how the pathogen was transmitted through different steps and processes, though it seems that field contamination followed by cross-contamination could be a plausible scenario. Cross-contamination during salad leafy vegetables processing has not been scientifically evaluated before. Risk assessment has been proposed as a discipline using scientific data to quantitatively evaluate microbiological risk in foods. Stochastic simulation modelling can be applied to estimate the consequence of highly variable processes, such as how microorganisms transmit along the food chain (Lammerding & Fazil, 2000). The present study aims at performing a stochastic model to evaluate *E. coli* O157:H7 cross contamination in a processing line for fresh-cut lettuce, estimating contamination levels at factory and identifying critical control points (CCPs) in the processing steps.

Materials and methods

A typical processing line of vegetables was modelled in this work by using probability distributions when appropriate. Certain simplifications are assumed to make modelling possible since the main purpose of the model is to be a practical tool to evaluate possible interventions and control measures reducing risk by *E. coli* O157:H7 in vegetable processing plants. The model was developed on the basic flow diagram of production of fresh-cut vegetables shown in Figure 1

Modeling transfer

Transfer data for *E. coli* O157:H7 were obtained in our laboratory simulating cross-contamination at different steps in a processing line for fresh-cut lettuce (shredding, belt, flume, shaker, and centrifuge) (Buchholz et al., 2008). Cross contamination took place during processing from a batch of inoculated cored heads of iceberg lettuce at 3 different levels of *E. coli* O157:H7 (6, 4, and 2 log cfu/g) to a batch of non-inoculated cored heads of iceberg lettuce. In short, inoculated lettuces entered the processing line followed by non-inoculated lettuce and then, a new batch of non-inoculated lettuce was processed. In order to quantify transfer, samples were taken from equipment surfaces after processing inoculated lettuces, and from processed non-inoculated lettuces. Transfer of bacteria involved the following six transfer scenarios: product-to-water,

product-to-equipment, water-to-equipment, and all three reverse transfers.

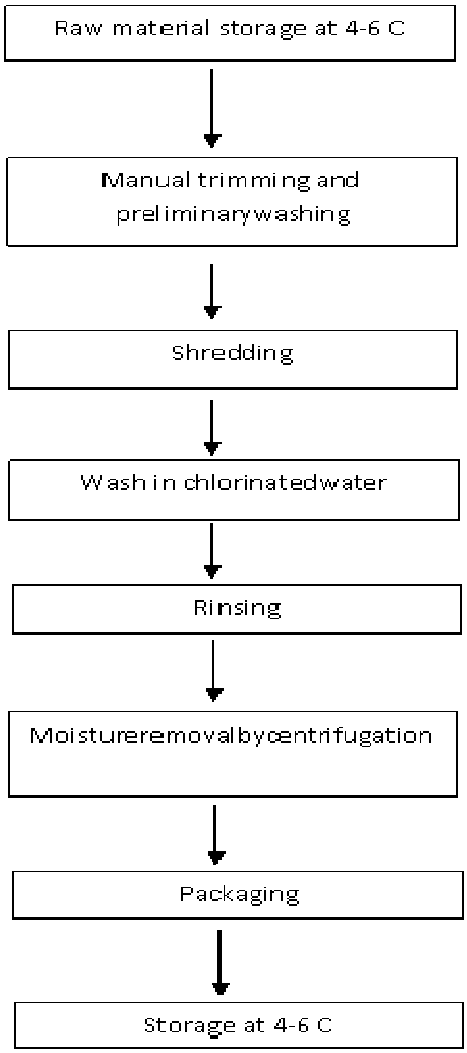

Figure 1. The basic scheme of production of minimally processed vegetables.

As transfer from contaminated surfaces to non-inoculated lettuce was unable to be estimated individually at each step, reverse transfers had to be modelled as an overall transfer coefficient accounting for combined transfer from contaminated equipment and water to non-contaminated lettuce. For that, transfer calculations were performed based on the overall contamination on surfaces and concentration on cross-contaminated lettuce after processing. Transfer coefficients were estimated exclusively using experimental data obtained at low contamination levels (2 log cfu/g) since they are expected to be more similar to reality. Probability distributions were fitted to these experimental data in order to capture both, the variability inherent to transfer events and the uncertainty derived from microbiological analysis (Perez-Rodriguez et al., 2008).

Probabilistic cross contamination model

Based on transfer coefficients distributions, a model was constructed in Excel which simulated the contamination processes from contaminated lettuces to non-contaminated lettuces because of cross-contamination at processing line (Figure 2).

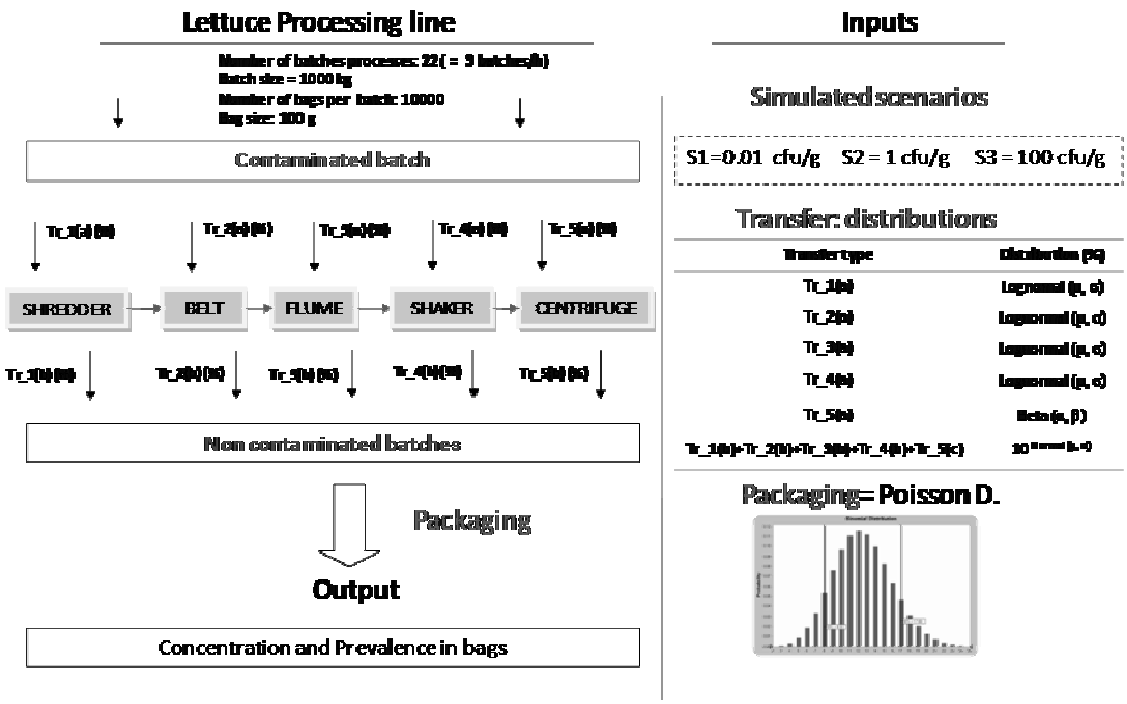

Figure 2. General scheme of the probabilistic model accounting for *Escherichia coli* O157:H7 transmission during processing of fresh-cut lettuce. The model describes cross-contamination from a contaminated lettuce batch to non-contaminated lettuce batches through equipment and processing water during processing. Cross contamination is modelled by combining probability distributions of transfer coefficients which describe bacterial transfer from product to equipment surfaces (Tr(a)) and from surfaces to product (Tr(b)), considering different initial levels for the contaminated lettuce batch entering the factory (S1= 0.01 cfu/g, S2= 1 cfu/g and S3=100 cfu/g). Concentration variability in cross-contaminated fresh-cut lettuce bags is described by a Poisson process.

In order to obtain comparable data among different simulations, model process parameters were fixed to: 22 batches processed per day (at 3 batches/h); Batch size: 1000 kg; Bag size: 100 g; Number of bags per batch: 10,000.

The probabilistic model assumed that the contaminated batch could enter the processing line at any time during production being an uncertainty source in the model. Due to the lack of data on prevalence and concentration of the pathogen, initial contamination for this contaminated batch in the model was simulated by 3 different potential contamination levels: Low level (S1): 0.01 cfu/g; Medium level (S2): 1 cfu/g; High level (S3): 100 cfu/g.

Model simulation comprised 22 batches which corresponded to a day of production. When the contaminated batch entered the processing line, which was modelled by a uniform distribution describing the position of the contaminated batch during processing (i.e. batch from 1 to 22), the number of cells transferred to the equipment (i.e. conveyor belt, shredder, flume, shaker table and centrifuge) and processing water was simulated by using the transfer coefficients described above. Concentration in the contaminated lettuce batch after processing was calculated by subtracting the number of cells transferred to equipment and water from the initial levels of *E. coli* O157:H7 in the contaminated batch at the different scenarios mentioned above (S1, S2 and S3). Then, cross-contamination in the subsequent batches was estimated by applying the overall transfer coefficient described above based on total number of microorganisms along equipment and water (i.e. total transfer from water and equipment to processed lettuce).

Table 1. Main inputs and models included in the cross-contamination model

Input	Model	Parameters	Source
Survival on equipment	*$\log(cfu)_s=\log(cfu)_{s-1}-(t_s/b)^a$	a= 0.17; b=0.02	unpublished data
Removal by washing	Uniform (min,max)	min=1 ; max=1.5	Han et al. (2002); Singh et al. (2002a);
Reduction in water by chlorine	**$\log(cfu)_f=\mu\cdot\log(cfu)_i+1$	μ=-0.02	unpublished data
Reduction on produce by chlorine	Uniform (min,max)	min=0.20; max=0.80	Singh et al. (2002b); Marks et al. (2009)
Reduction on produce by irradiation	$\log(cfu)_f=-(1/D)\cdot\log(cfu)_i$	D =0.11 KGy	Niemira (2008)
Bacterial distribution in processed lettuce	Poisson (S*C)	S= bagged sized C= cfu_f/g	Perez-Rodriguez et al. (2007)

*$\log(cfu)_s$: concentration on equipment at any time (t_s); *a* and *b* are fitting parameters based on Weibull model; t_s is the time that microorganisms are on equipment surfaces (h).
**μ is the reduction rate of *Escherichia coli* O157:H7 in chlorinated water; $\log(cfu)_f$: concentration on lettuce after treatment; $\log(cfu)_i$: concentration on lettuce before treatment

The initial number of *E. coli* O157:H7 cells on the processing line should decrease along processing as cells are removed from the contaminated equipment and water when transferred to each batch by cross-contamination. Therefore, to describe the *E. coli* O157:H7 cells remaining on equipment and water after processing each batch, the amount of cells transferred to each batch during production was subtracted from the previous concentration levels on equipment surfaces and water. Furthermore, the pathogen survival on surfaces was modelled to more accurately simulate transfer from contaminated equipments to non-contaminated lettuce over different time periods. For this, in-house experimental data were used (see Table 1).

There exists little experimental and published information concerning how cross-contaminated microorganisms are distributed in processed lettuce. In this study, we considered that processing water could disseminate cells uniformly on lettuce pieces during processing. To assess this hypothesis, the model was previously simulated assuming homogenous and non-homogenous cells distribution by using a Poisson distribution, and negative-binomial distribution, respectively. Results indicated there was no significant difference between both approaches (data not shown).

Therefore, packaging of processed lettuce was modelled assuming that *E. coli* O157:H7 was distributed homogeneously on both, the contaminated lettuce entering processing line and cross-contaminated lettuce during processing. Hence, the Poisson distribution was used to describe *E. coli* O157:H7 concentration in the product bags (Table 2). Results from the validation process, performed in this study, could be helpful to evaluate the suitability of this assumption. Nevertheless, model estimations should be carefully interpreted as the uncertainty derived from such an assumption.

The impact of different sanitation regimes, disinfection processes (i.e., irradiation and chlorination treatments), and sampling plans at different steps in the processing line (e.g., shredder, shaker table, and conveyor) on the prevalence and concentration of *E. coli* O157:H7 in the bags of product could also be evaluated using the model (Table 1). In the case of disinfection processes, chlorinated water at different levels (0-200 ppm) was simulated taking into account the decontamination effect on microorganisms in both, water and lettuce surface by using models presented in Table 2. For irradiation treatment, a log-linear model was considered as shown in Table 2, accounting for inactivation by irradiation levels from 0.25 to 1.5 KGy. Sanitation regimes were models on basis of percentage of equipment surface accessible to a cleaning and disinfection procedures. This procedure was supposed to be 100 % effective at removing the pathogen, when applied.

Table 2. Main statistics of transfer data set and fitted probability distribution

Transfer (%) at low level	Maximum	Minimum	Mean	Distribution
Produce-Shredder	0.02	0.00	0.02	Log-Normal
Produce-Flume	0.02	0.00	0.01	Log-Normal
Produce-Shaker	0.02	0.00	0.01	Log-Normal
Produce-Centrifuge	0.08	0.01	0.04	Beta
Produce-Conveyor	0.24	0.00	0.10	Log-Normal
Produce-Water	10.46	0.00	8.79	Beta
Equipment-Produce	18.83	9.90	15.33	Log-Normal

Simulation and model validation

The model was developed containing two separate dimensions, variability and uncertainty (i.e. a two-dimensional model). In general, uncertainty refers to the lack of knowledge on a measurable property of a system, while variability is the natural variation present in a system or inherent to a phenomenon. Unlike variability, uncertainty can be reduced, for example, by increasing the number of measurements of the system. In the model, the time when the contaminated enters the processing line was considered as uncertainty as it is unknown how often a lettuce batch entering the factory is contaminated. Also, transfer coefficients distributions were simulated as uncertainty sources in the model. Simulation was performed with 10000 variability iterations and 10 uncertainty realizations. The simulation of the variability dimension was carried out by applying the Latin Hypercube technique implemented in @Risk Professional© software (Palisade, Newfield, NY). On the other hand, the uncertainty dimension simulation was performed by using a simple random sampling implemented in Excel® for those variables representing uncertainty. The simulation output provided an estimate of *E. coli* O157:H7 populations in commercially bagged product. In order to validate the model, predicted concentration values in bagged lettuce, after processing, were compared to experimental values obtained in laboratory settings not included in the model. For that, Standard Error of Prediction (SEP) was used applying the following formula:

$$SEP = \frac{100}{\overline{Y}} \cdot \sqrt{\frac{\sum_{i=1}^{n} (X - Y)^2}{n}} \qquad \text{eq. 1}$$

Where X is the predicted value, Y the observed value, $\overline{Y}$ mean for the observed values and n, the number of samples.

Statistical analysis

Descriptive statistics of the data obtained during simulation were calculated using Microsoft Excel TM. Differences between scenarios were examined by performing non-parametric variance analysis (Kruskal-Wallis test) ($P < 0.05$) on simulated prevalence and concentration in bagged products. A Sheffe test ($P< 0.05$) was performed on the same data set, which allowed the formation of homogenous groups by an association of scenarios with concentrations statistically similar. This analysis was carried out using Statistica 5.5 Software (Statsoft ©, Tulsa, USA).

Results and Discussion

Probability distributions for *E. coli* O157:H7 transfer and model validation

Different probability distributions were fitted to observed transfer data and the goodness of fitting was evaluated by Kolmogorov-Smirnov test, Chi-Squared Statistic and visual analysis, (Cullen & Frey, 1999). The most suitable distributions to describe transfer data were Beta and Log normal distributions. Distributions parameters fitted to experimental transfer data are presented in Table 2. These results show that higher transfer occurred from produce to processing water and from equipment to lettuces. In order to make results comparable, validation was performed by using observed and predicted data obtained at high level, i.e., when lettuces were artificially contaminated with 100 cfu/g in the experimental assay and S3 was simulated in the model. By using these data, the SEP (Standard Error of Prediction) was estimated, which ranged 0.00-

35%. Since the SEP= 35 % (i.e. approximately 0.5 log shift) is within the typical plate count error, the cross contamination model could be satisfactorily validated based on this criterion.

Simulated model

Figure 3 shows uncertainty on the total percentage of *E. coli* O157:H7 transferred from initially contaminated lettuce to non-contaminated lettuce in the processing line for scenario 2 (S2= 1 cfu/g). These values ranged between 0% and 0.32 %. The value 0 % was because the contaminated batch entering to processing line was the last one before the final production of the day. Similar total transfer percentages could be observed for S1 and S3. These results suggest that cross contamination occurred at relatively low levels. However, the increase of prevalence derived from cross-contamination (percentage of cross-contaminated bags) showed higher levels. Thus, for example, for S2, the uncertainty range on the increase of prevalence (from cross contamination) was between 0 and 4.32 % (9646 bags), with a mean value of 3.05 % (6711 bags) (see Table 3). For S1 and S3, the average prevalence levels which resulted from simulation were 0.09 and 13.39 %, respectively (see Table 3).

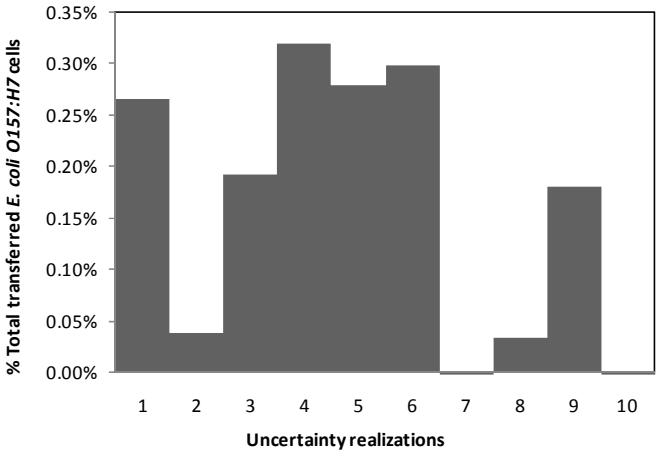

Figure 3. Percentage of transferred cells of *Escherichia coli* O157:H7 from contaminated lettuce to non-contaminated lettuce during production (after 22 batches) for 10 uncertainty realizations.

The ANOVA analysis performed on simulated data revealed that the initial level in the contaminated batch (S1; S2 and S3) did not influence significantly (p=0.4) the concentration levels in bags derived from cross-contamination which were always below 10 cfu/bag (Figure 4). In turn, for prevalence, there were significant differences (p< 0.001) at the different levels simulated (S1; S2 and S3). Figure 5 reveals that the increase of number of bags contaminated by *E. coli* O157:H7 through cross-contamination decreased logarithmically along the production for the 3 simulated scenarios. In S1(S1=0.01 cfu/g) cross-contamination occurred only during the first hour (3 batches/h), immediately after the originally contaminated batch entered the processing line, showing a pronounced drop of prevalence to 0 % as shown in Figure 5. However, even at this low level, bags were cross-contaminated sporadically, resulting in very low concentration (mean: ≤ 2 cfu/bag) and prevalence levels (<1 %) (see Table 3). At medium contamination level (S2), cross-contamination remained high for the first two batches (10,000 cross-contaminated bags/batch), then the number of cross-contaminated bags dropped significantly, in the following batch, to 100 cross-contaminated bags/batch, followed by a much more gradual decline up to 10 cross-contaminated bags in the last batch (Figure 5). With the S3 scenario (100 cfu/g) there

was a gradual decline in the number of cross-contaminated bags along the whole production sequence from 10,000 to 1,000 cross-contaminated bags per batch, indicating that higher initial contamination numbers are more likely to persist in subsequent batches if not decontamination steps are used.

Interventions

In the present model, the effect of different intervention scenarios was evaluated: decontamination by irradiation, and by chlorination and then application of a sampling plan to test and remove contaminated batches after processing, together with cleaning and disinfection procedures.

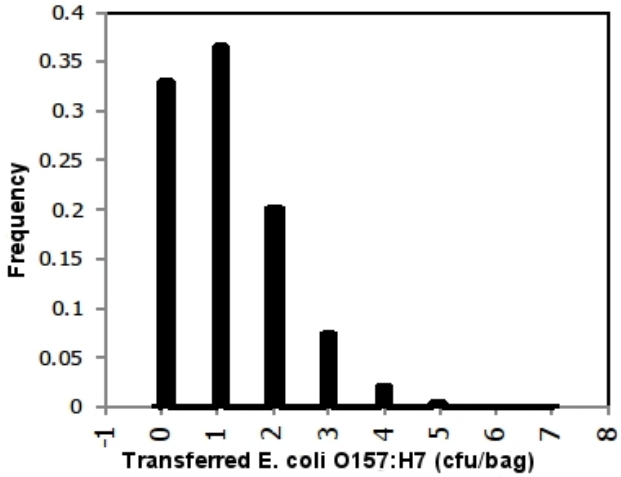

Figure 4. Example of the simulated distribution of *Escherichia coli* O157:H7 in bags in Scenario 3 (S3=100 cfu/g).

Chlorination

Chlorination is the most widespread disinfection treatment. Studies have reported reduction in the range 1-2.5 log CFU/g (Francis &O`Beirne, 2002, Legnani&Leoni, 2004, Han et al., 2002 and Behrsing et al., 2000). However, it is well-established that elimination of pathogens from the surface of vegetables by disinfection is limited and unpredictable (Gil et al., 2009, Nguyen-the & Carlin, 1994). Moreover, it has also been shown that the chlorine concentration does not necessarily kill bacteria, and after removing chlorine some can recover during the rinsing step (Legnani and Leoni, 2000). In the light of these facts, it seems clear that disinfection treatments can fail resulting in the presence of the pathogen in lettuce at the moment of consumption. Furthermore, recently, it has been suggested that *E. coli* O157:H7 could become internalized into plant tissues reducing thus the effectiveness of disinfection treatments (superficial) (Watchel et al., 2003).

Table3. Prevalence of cross-contamination bags at the different simulated scenarios

Scenario	Prevalence (% bags)					
	Baseline	Chlorination			Irradiation (0.5KGy)	Cleaning& disinfection
		50 ppm	100 ppm	200 ppm		
S1	0.09 (0.23)*	0.02 (0.10)	0.01(0.08)	0.001 (0.002)	no contamination	0.00002 (0.00005)
S2	3.05 (4.37)	0.20 (0.32)	0.16 (0.26)	0.14 (0.23)	0.0005 (0.001)	0.01 (0.04)
S3	13.39 (25.09)	4.00 (5.31)	3.78 (5.06)	3.28 (5.01)	0.03 (0.09)	0.07 (0.11)

*Mean (95th percentile).

The model was simulated including a decontamination step with chlorine at 50-200 ppm assuming that chlorine levels could be maintained constant during a day of production throughout the washing stages. As shown in Table 3, a noticeable reduction of cross-contaminated bags was obtained when 50 ppm of chlorine in water was simulated for the different concentration scenarios (S1-S3). In the case of S1, the average number of cross-contaminated bags was 0.02 % which means a reduction of 450 % cross-contaminated bags with respect to baseline model and, for S2 and S3, means of 0.20 and 4.00 % cross-contaminated bags were obtained, respectively which means a reduction of 1500 and 33 % of prevalent bags with respect to baseline model. At higher concentrations of chlorine (100-200 ppm), the reduction of the number of cross-contaminated bags was much lower for S2 and S3 as shown in Table 3. Even if chlorinated water was maintained at 200 ppm throughout the washing stages, *E. coli* O157:H7 was able to survive on lettuces at different contamination scenarios (S1=0.001%; S2=; 0.14%; S3=3.29%) (see Table 3). At high levels (S3), chlorination (200 ppm) was not as effective in reducing cross-contamination as at medium and low levels (S2 and S1), i.e., chlorination reduced S3 prevalence levels by about 340 % compared with 9000 % and 2200 % for S1 and S2 levels, respectively. Therefore, control measures to reduce transmission of the pathogen from farm environment to vegetables at harvest, retail and home could result in a minor incidence of the pathogen. In this sense, the application of guides, GMPs, etc. could help reducing the incidence of these contamination routes.

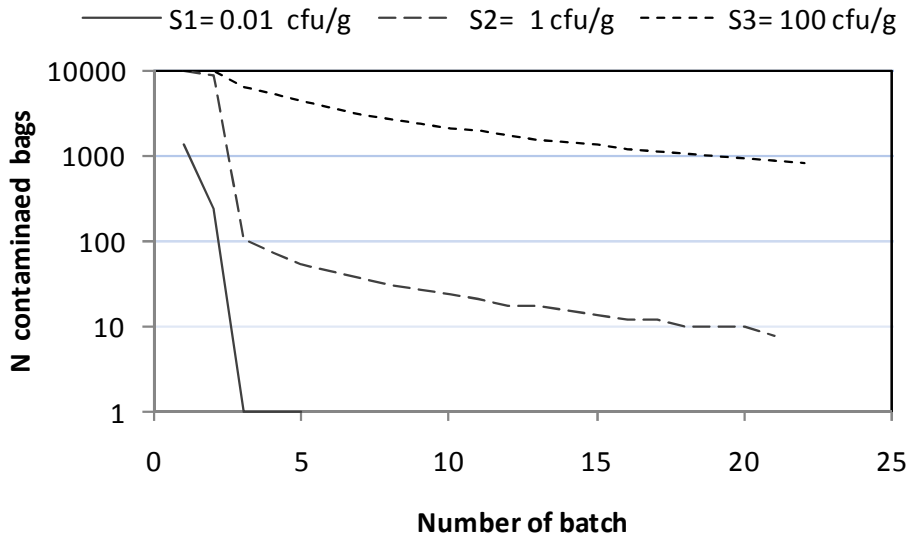

Figure 5. Simulated number of cross-contaminated bags along the production (22 batches) when one contaminated batch enters the processing line.

Irradiation

Chlorination has long been used in the produce industry, but has not been completely effective. Recently the United States Food and Drug Administration (FDA) approved the use of gamma irradiation on vegetables allowing irradiation levels up to 4KGy (USFDA, 2008). However, sensory characteristics in irradiated vegetables can be affected at irradiation levels above 0.5KGy (Niemira, 2008, Niemira et al., 2002, Foley et al. 2002), and so only lower irradiation levels could be used practically. In the model,

by applying 0.5KGy (borderline for sensory acceptance), in S3**,** resulted in a prevalence average in final bags of 0.03%. For S2, the prevalence was reduced by up to an average value of 0.0005% (Table 3). For S1, applying an irradiation treatment of 0.5KGy on the final product resulted in all bags being non-contaminated, i.e., eliminating fully contamination in both the originally contaminated lettuce entering the processing line and cross-contaminated lettuce occurred during production. However, when lower values (<0.5KGy) were simulated, *E. coli* contamination could be still found in a very small number of bags (≤0.15%).

When a combination of chlorination (200ppm) and irradiation (0.5KGy) used sequentially for lettuce contaminated at high level (S3), was simulated, the concentration was ≤ 2 cfu/bag, and prevalence ranged between 0.01 and 0.14 % (mean, 0.06 %). For medium level (S2), the combination of both inactivation treatments resulted in only one cross-contaminated bag after 10 uncertainty realizations of the model. This very low value could be considered to be 0% practically. Therefore, combining both decontamination steps could be an effective intervention to practically eliminate cross-contamination at medium contamination levels (S2).

Pathogen testing

The last intervention simulated was the effect of a 2-class attributes sampling plan with n≤ 10 and c= 0 (sample size = 25g) in the different contamination level scenarios (S1, S2, and S3) to allow testing and rejection of positive batches of final product. As the distribution of *E. coli* O157:H7 in processed lettuces was assumed to follow a Poisson process, the exponential distribution can be applied as described by van Schothorst et al. (2009) to estimate the probability of detecting one or more contaminated samples of bagged lettuce taken at the end of production according the different sampling plans. The exponential distribution used was defined as $p(x\geq 1) = 1-\exp(-C\cdot S)$, being C the concentration (CFU/g) and S the sample size (g) (Perez-Rodriguez et al., 2007).

Percentages of rejected contaminated bags were calculated with respect to the baseline model according to following equation:

$$P(\%)_r = \frac{\sum_{D=1}^{n} Number\ of\ \ rejected\ bags}{\sum_{T=1}^{n} Number\ of\ \ contaminated\ bags} \times 100 \qquad \text{eq.2}$$

Where $P(\%)_r$ corresponds to percentage of rejected contaminated bags with respect to the baseline model; D=1…n denotes the number of detected contaminated batches; and T= 1…n denotes the number of total contaminated batches. Note that *number of rejected bags* in eq. 2 refers to the number of bags (i.e. 10,000) in rejected batches.
These percentages are represented in Figure 6 for different n simulated (1, 3, 5, and 10). Results indicated that only a sampling plan with n=10 in both, S2 and S3 could result in most of contaminated bags rejected (~100 %). Conversely, sampling plans were not effective when the product was contaminated at low (S1), and no positive bags could be detected along different simulations. Surprisingly, sampling plans applied on the S2 scenario (i.e., medium contamination entering processing line) resulted in higher percentages of rejected contaminated bags when compared to S3 (i.e., high contamination level). This unexpected result was related to the fact that concentration level in final product was not affected by the initial contamination level (i.e., S1, S2,

and S3). Therefore, as S3 was associated with higher prevalence compared to S2, when positive batches were not detected because of low concentration, this yielded higher number of non- rejected contaminated bags in S3.

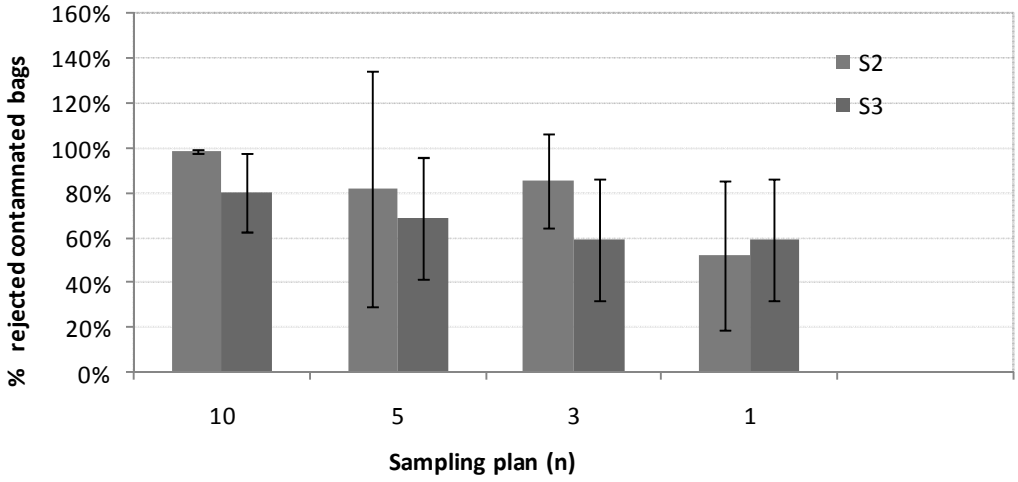

Figure 6.Simulated rejected cross-contaminated bags along production (22 batches) when sampling plans of two classes are applied with different number of samples (n= 1, 3, 5, and 10)

For a sampling plan with n=5 in S3 (high level), the prevalence was reduced by up to 9.2% (average) which meant an average reduction of 68 % out of contaminated bags on the baseline model, if the contaminated batches were detected and destroyed. Although average values were quite similar between n=5 and n=3, especially in S2 (1 cfu/g), simulations using n =5 led most often to higher number of rejected contaminated bag compared to n=3. Likewise, for high levels (S3), sampling plans with n =1 and 3 showed similar probability of detecting *E. coli*. However, this fact could not be observed for S2, in which sampling plans using n=3 were more efficient detecting contaminated bags. In summary, results indicated that sampling plans can lead to an effective reduction of *E. coli* O157:H7 risk in processing line, especially at medium level, but it becomes ineffective when low contaminations are considered which is the most probable situation under most field and processing conditions. Performing a sampling plan (n=5) together with chlorination treatment led to a significant prevalence reduction only in S3, in which prevalence reached a mean value of 1.53%. Similarly, the use of a sampling and testing plan had a positive effect when applied after an irradiation treatment at 0.1KGy, resulting in a mean prevalence of 0.8 %; however, at higher irradiation levels, sampling did not have significant effect on prevalence.

Cleaning and disinfection

It is reasonable to expect that after a contaminated batch is detected, sanitation procedures would be applied to remove bacterial contamination from processing line. In order to assess the impact of cleaning and disinfection on number of cross-contaminated bags, an efficacy rate (%) for sanitation procedures was applied to different steps along the processing line considering different contamination level scenarios i.e., S1, S2, and S3. The efficacy rate was here considered as percentage of contaminated contact zones and water which is correctly treated which means that cleaning and disinfection are completely effective in removing or eliminating microorganisms. For example, if efficacy rate is equal to 50 %, it would mean that the 50% of contaminated equipment and water have been cleaned and disinfected eliminating any bacterial pathogens

present. In simulation, a maximum efficacy rate of 99 % was regarded since it would be expected that all zones in the processing line (e.g., grooves, screws, joints, etc.) could be efficiently treated. Results indicated that when cleaning and disinfection was carried out with maximum efficacy rate, cross-contaminated bags were still found, in simulation, even though the number of cross contaminated bags was very small (Table 3). It is remarkable that, for S1, with an initial low concentration, cross-contamination after cleaning and disinfection was simulated to occur sporadically during the processing of the 22 batches (0.00002 %), in which each contaminated batch presented only one potentially contaminated bag.

Conclusions

The present study applies a simple modelling approach to provide useful information to processing managers in the leafy green industry. Although, as much accuracy as possible is preferred when data are modeled or quantitative risk assessment studies are performed, simple models with limited data can help make decisions and give response to important risk management questions. As shown in this work, comparative analysis between different intervention and mitigation strategies can be carried out without complex modeling of detailed processing lines, and guidance for priorities can be identified.

Unfortunately, we do not know what the concentration of *E. coli* O157:H7 is when it enters the processing line, but we believe it can be as high as the S3 level on rare occasions, e. g. animal feces on several lettuce plants. Even if lower levels are more typical, once *E. coli* O157:H7 enters in a processing line, cross contamination can occur. Control measures, such as chlorination, irradiation and frequent *E. coli* testing can significantly reduce the likelihood of cross-contamination, especially if they are all considered together. At low contamination levels, the decontamination steps here simulated were especially effective at reducing cross-contamination. Furthermore, results demonstrated that irradiation (0.5 KGy) was a more effective decontamination step in reducing prevalence than chlorination under the same simulated conditions, although there are economic and marketing issues relating to this technology that it is not currently used by the industry. In these scenarios it was found that even with all the possible interventions, there is always small probability that *E. coli* O157:H7 can contaminate a bag leaving the processing facility for distribution and sale.

Acknowledgements

This work was partly financed by United States Department of Agriculture with the Grant GEO-2007-01899, MICINN AGL2008-03298/ALI, the Excellence Project AGR-01879 (Junta de Andalucía) and by the Research Group AGR-170 HIBRO of the “Plan Andaluz de Investigación, Desarrollo e Innovación” (PAIDI).

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Chapter II: "Electrochemical disinfection: An efficacy treatment to inactivate *E. coli* O157:H7 in process wash water containing organic matter"

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Published in: Food Microbiology 30, 146-156, 2012

Abstract

The efficacy of an electrochemical treatment in water disinfection, using boron-doped diamond electrodes, was studied and its suitability for the fresh-cut produce industry analyzed. Tap water (TW), and tap water supplemented with NaCl (NaClW) containing different levels of organic matter (Chemical Oxygen Demand (COD) around 60, 300, 550±50 and 750±50 mg/L) obtained from lettuce, were inoculated with a cocktail of *Escherichia coli* O157:H7 at 10^5cfu/mL. Changes in levels of *E. coli* O157:H7, free, combined and total chlorine, pH, oxidation-reduction potential, COD and temperature were monitored during the treatments. In NaClW, free chlorine was produced more rapidly than in TW and, as a consequence, reductions of 5 log units of *E. coli* O157:H7 were achieved faster (0.17, 4, 15 and 24 min for water with 60, 300, 500 and 750 mg/L of COD, respectively) than in TW alone (0.9, 25, 60 min and 90 min for water with 60, 300, 600 and 800 mg/L of COD, respectively). Nonetheless, the equipment showed potential for water disinfection and organic matter reduction even without adding NaCl. Additionally, different mathematical models were assessed to account for microbial inactivation curves obtained from the electrochemical treatments.

Introduction

In the last three decades, several food-borne outbreaks have been associated with the consumption of leafy vegetables contaminated with *Escherichia coli* O157:H7 (Ackers et al., 1998; Rangel et al., 2005; Söderström et al., 2005), which shows the pathogen could attach to lettuce leaves, contaminating the final product (i.e. fresh-cut vegetables) and causing illness (Kroupitski, et al., 1011). However, there is scarce knowledge on how the pathogen is transmitted through different processing steps, though it seems that cross-contamination during washing at the factory could have an important role. Disinfection of process water is a critical step in reducing cross contamination, however, studies show that in many cases chlorinated water is not fully effective in reducing the pathogen, thereby enabling cross contamination during the industrial process (Zhang et al. 2009). Sodium hypochlorite continues to be widely used by the fresh-cut industry for washing and disinfecting (Lee &Baek, 2008; Gopal et al., 2010). However, currently, extensive research is being carried out aimed at investigating the effectiveness of alternative disinfection treatments in eliminating *E. coli* O157:H7 contamination during fresh-cut produce processing. On the other hand, the fresh-cut produce industry needs to reuse water in order to be sustainable from both the economic and the environmental points of view (Casani et al., 2005; ILSI, 2008; Selma et al., 2008; Ölmez & Kretzschmar, 2009). Hence, it is very important to find technologies suitable for recycling process water, with the purpose of reducing water consumption and wastewater discharge. Such technologies should be powerful enough to provide water that does not contain microorganisms or toxic substances in amounts that can influence food safety (EU, 1998; EU, 2004) and that could be used without affecting the

wholesomeness of the product, but that would not need to be necessarily of potable quality.

Previous studies confirmed that the main function of sanitizers is to maintain the microbial safety of the process water avoiding cross contamination (Parish et al., 2003; Gil et al., 2009; López-Gálvez et al., 2010a). Unfortunately, chlorine reacts with the organic matter present in the water and, as a consequence, formation of potentially harmful chlorinated by-products in the process water can occur (López-Gálvez et al., 2010b). Hence, the reuse of process water (without any previous treatment) would lead to the accumulation of organic matter and disinfection by-products. In this sense, it is crucial to develop new technologies which enable the reduction of the level of chlorine required for disinfection and reduce the level of organic matter in the process water, with the aim of reducing the formation of disinfection by-products.

Electrochemical treatment of water has shown potential for the disinfection and improvement of physicochemical quality of different types of water, such as drinking water (Martínez-Huitle & Brillas, 2008), and both industrial and domestic wastewaters (Chen, 2004; Ongeng et al, 2006; Anglada et al, 2009; Schmalz et al., 2009; Poyatos et al., 2010). The utilization of this technology in the fresh-cut processing line could help to reduce the chlorine requirements in the washing tank while helping to maintain a reasonable safety level of fresh-cut products by avoiding cross-contamination (López-Gálvez et al., 2010a). Electrochemical treatment is a very powerful technology with two mechanisms of action: direct oxidation at the anode surface and indirect oxidation in the bulk solution by oxidants produced from the substances present in the water (Anglada et al, 2009). This technology can be used to mineralize organic matter and disinfect water even in the absence of chloride and, therefore, without formation of free chlorine and its by-products (Kerwick et al., 2005; Polcaro et al., 2007; Jeong et al., 2009; Barashkov et al., 2010). Another advantage of electrochemical disinfection is that the damage produced to the bacterial cells is more severe than that produced by pure chemical disinfection with chlorine (Diao et al., 2004; Wang et al., 2010). Different factors have to be taken into account in order to adjust the electrochemical disinfection design for a specific application. The electrode material is one of the most important factors to be considered. Boron-doped diamond (BDD) electrodes are less effective than other kinds of electrodes in the production of free chlorine from chloride present in the water. On the other hand, BDD electrodes have shown higher potential compared with other types of electrodes to produce reactive oxygen species (ROS) such as hydroxyl radicals, and other non-chlorine-based oxidants (Martínez-Huitle & Brillas, 2008; Cañizares et al., 2009; Jeong et al., 2009; Polcaro et al., 2009). Thus, BDD electrodes would be more suitable electrodes than others for the disinfection of process wash water with low chloride content. Therefore, the aim of the present study was the evaluation of the efficacy of electrochemical treatment using BDD electrodes for the inactivation of pathogenic microorganisms in fresh-cut produce wash water, using *Escherichia coli* O157:H7 as a model, and for the reduction of the amount of organic matter in water. Additionally, non-linear regression analyses were used to predict the experimental reductions of *E. coli* O157:H7 in vegetable wash water.

Materials and methods

Bacterial strains and inoculum preparation

A five-strain cocktail of *Escherichia coli* O157:H7 strains (CECT 4267, 4076, 4782, 4783, and 5947), provided by the Hibro Group from the University of Cordoba (Spain),

was used in the study. Cultures were rehydrated in Brain Heart Infusion broth (BHI, Oxoid, Basingtoke, United Kingdom). Nalidixic acid-resistant (NalR) *E. coli* O157:H7 cultures were obtained by consecutive 24-h transfers of BHI cultures to BHI with increasing concentrations of nalidixic acid (Nal) (Merck, Darmstadt, Germany) until strains were resistant to 50 μg of Nal per mL. NalR *E. coli* O157:H7 cultures were consecutively subcultured twice in 5 mL of BHI supplemented with nalidixic acid (Nal+, 50 μg/mL) at 37 °C for 20 h. After the second incubation, cultures were mixed, equal volumes of cell suspensions were combined to give approximately equal populations of each culture (10^8cfu/mL). Then, the cocktail was washed three times by centrifugation (4100 g) at room temperature for 10 min with 0.1% $NaHCO_3$, and the final cell pellets were suspended in 0.1% $NaHCO_3$ obtaining an inoculum with a level of 10^9cfu/mL, approximately. Final concentrations of the inoculum solutions were confirmed by plating on chromocult coliform agar (Merck, Barcelona, Spain) supplemented with Nal (50 μg/mL).

Preparation of water with high chemical oxygen demand

Iceberg lettuce (*Lactuca sativa L.*) was purchased from a local wholesale market in Murcia (Spain) at the day of harvest and transported within 15 min under refrigerated conditions to the laboratory. Outer leaves were manually removed and discarded while internal leaves were cut into 3 cm pieces. Afterwards, 67 g of lettuce were placed into stomacher bags (Seward Limited, London, UK), 200 mL of water (tap water or ultra pure water depending on the experiment) were added, and the mixture was homogenized for 2 min in a stomacher (IUL Instruments, Barcelona, Spain). Finally, water was filtered through a nylon mesh with gaps of 0.5 mm, in order to avoid obstruction of the electrolytic cell.

Table 1. Characteristics of the model waters used in the experiments.

Type of water	Chemical Oxygen Demand (COD) (mg/L)	Added salts
Ultra pure water (UPW)	800	$NaHCO_3$ 388 mg/L & Na_2SO_4 627 mg/L
Tap water (TW)	60, 300, 600 & 800	None
Tap water + NaCl (NaClW)	60, 300, 500 & 750	NaCl 1000 mg/L

Preparation of fresh-cut produce processing model waters

Three types of model waters were used: (1) ultra pure water (UPW) (Millipore system, Millipore Corp, Bedford, MA, USA) supplemented with inorganic salts, (2) tap water (TW), and (3) tap water supplemented with 1 g/L NaCl (NaClW). Table 1 shows, in detail, the characteristics of the model waters used in the experiments. NaHCO3, Na2SO4 and NaCl were obtained from Sigma-Aldrich (St Louis, MO, USA). The main physico-chemical characteristics of the TW used in the experiments were analyzed by the ionomic laboratory from CEBAS-CSIC. Elements were determined by plasma optical emission spectrophotometry (ICP OES thermo-IRIS Intrepid II XDL Duo). Ions were determined by ion chromatography (Metrohm) while total carbon (TC), total organic carbon (TOC), inorganic carbon (IC) and total nitrogen (TN) were determined using a 2400 CHN Elemental Analyzer, (Perkin Elmer). Table 2 includes detailed

information about the physicochemical characteristics of the TW. In order to obtain water with different levels of COD for the electrochemical treatment, UPW, TW, or NaClW were mixed with the adequate volume of water with high COD.

Table 2. Physicochemical characteristics of the tap water used in the experiments. TOC: total organic carbon; IC: inorganic carbon; TC: total carbon; TN: total nitrogen.

Elements		Ions		Other parameters	
Ca (mg/L)	73.46	F^- (mg/L)	0.16	TOC (mg/L)	0.00
Fe (mg/L)	0.16	Cl^- (mg/L)	115.85	IC (mg/L)	46.38
K (mg/L)	6.93	Br^- (mg/L)	0.12	TC (mg/L)	46.08
Mg (mg/L)	47.05	NO^{3-} (mg/L)	4.17	TN (mg/L)	1.46
Na (mg/L)	135.40	SO_4^{2-} (mg/L)	266.62	Conductivity (µS)	1417.00
S (mg/L)	109.50			pH	8.20

Electrochemical equipment and treatments

Disinfection experiments were performed using a lab scale treatment system provided by Adamant Technologies (La Chaux-de-Fonds, Switzerland). The treatment system included: power supply, control board, pump, treatment tank, flow-meter, pipes, and an electrolytic cell. Refrigeration of the treated water was done by pumping cold water through stainless steel pipes immersed into the treated water. Two kinds of electrolytic cells were tested during the experiments: Diacell 101 and Diacell401. In both cases anodic and cathodic compartments were not separated. Diacell 101 was equipped with one cell containing one BDD cathode and one BDD anode with an overall effective anode surface area of 67 cm^2. On the other hand, Diacell 401 was comprised of four cells of the same characteristics placed in parallel. In both cells, the electrodes had an inter-electrode gap of 1 mm.

Different sets of electrochemical disinfection experiments were carried out. First, preliminary tests were done in order to determine: (1) The optimum DiaCell configuration (101 or 401) for the treatment of model waters, and (2) efficacy of DiaCell 401 for the disinfection of water in the absence of chloride (using UPW). Secondly, disinfection tests were repeated three times on different dates, treating TW and NaClW with DiaCell 401 to determine the applicability of this technology for the disinfection of fresh-cut produce wash water. In all cases pH of water was adjusted to 6.5 before the treatments using citric acid in order to improve chlorine disinfection efficacy. Temperature of water at the start of the treatments was around 5 ºC, and the maximum variations during the treatments were of ±2 ºC. Model waters were inoculated with the NalR *E coli* O157:H7 cocktail at an inoculum level of approximately 5 log cfu/mL just before the beginning of the treatment. A volume of 5 L of inoculated model water was placed in the polypropylene tank (capacity: 12 L) and pumped through the electrolytic cell returning to the tank and starting the cycle again. Movement of water was obtained by a pump with a volumetric flow rate fixed at 800 L/h. Amperage was kept during the experiments at a level of 6.4 A (1.6 x 4) for DiaCell 401 and 14.5 A for

DiaCell 101. These two used amperages were different because maximum applicable amperage was higher for 101 than for 401, and tests were designed to obtain the maximum effectiveness of each configuration. As a consequence, current density applied was 24 mA/cm^2 when using DiaCell 401, and 216 mA/cm^2 in DiaCell 101 treatments. During the experiments, polarity of the electrodes was inverted each 20 minutes, to avoid fouling of the surface of electrodes.

Microbiological analyses

Changes in levels of NalR E *coli* O157:H7 were measured at different time intervals. For that, 10 mL-samples were taken from treated water and transferred into tubes containing sodium thiosulphate in order to neutralize residual oxidants present in the sample. Then, samples were diluted when needed, using buffered peptone water (Scharlau, Barcelona, Spain), and plated in Chromocult coliform agar supplemented with Nal (50 μg/mL) using a spiral plater (model WASP, DW scientific, Shipley, UK). Plates were incubated at 37 ºC for 24 h before counting in an automated plate counter (ProOL, Synoptics, Cambridge, UK).

Physicochemical analyses

Changes in levels of free and total chlorine (mg/L), pH, oxidation-reduction potential (ORP, in mV), temperature (ºC), and COD (mg/L) were measured at different time intervals depending on the type of water treated. Additionally, in the case of the UPW treatment, level of hydrogen peroxide (H_2O_2) was also monitored. Temperature, ORP, and pH were measured using a multimeter pH & Redox 26 (Crison, Barcelona, Spain). Free and total chlorine were determined based on the N,N-diethyl-p-phenylendiamine (DPD) method (APHA, 1998) using the Spectroquant NOVA 60 photometer (Merck, Darmstadt, Germany). Chemical Oxygen Demand (COD) was determined by the standard photometric method (APHA, 1998) using the Spectroquant NOVA 60 photometer. Hydrogen peroxide level was measured by using the Merckoquant Peroxide Test (Merck, Darmstadt, Germany).

Statistical analysis and data modeling

Experiments to evaluate the inactivation by electrochemical treatment of *E. coli* O157:H7 inoculated on water were performed in triplicate per type of water and COD load in different dates. For data analysis, *E. coli* O157:H7 count data obtained at different contact times for the different experimental conditions were log-transformed (log cfu/mL), and mean and standard deviation were estimated. Mean counts of *E. coli* O157:H7 were plotted versus contact time in Excel (Microsoft Corporation) spreadsheet and then were analyzed by non-linear regression to assess different inactivation kinetic models (Table 3). The statistics RMSE, R^2 and adjusted R^2, all with a significant level of $P<0.05$, were used to determine the best model for fitting where a RMSE closer to 0 indicates a better fit (Ratkowsky, 2002). All models were fitted to data by using the curve fitting toolbox provided by the GInaFiT (Geeraerd et al., 2006a & 2006b). For each model, the three sets of experimental data were used to estimate, via the Root Mean Square Error (RMSE), R^2 and adjusted R^2, how well the model predicts this experimental data.

Table 3. Description of the disinfection kinetic models used to analyze the experimental data obtained in this study.

Model Name	Model	Model Parameters	References
Log linear	$N= N_0 * exp(-k * t)$	*k*	*Bigelow &Esty, 1920*
Log linear + shoulder	$N= N_0 * exp(-k * t) * (exp(k * Sl))/(1+(exp(k * Sl) - 1) *exp(-k*t)))$	*k, Sl,*	*Geeraerd et al. 2000*
Log linear + tail	$N= (N_0- N\_res) * exp(-k * t) + N\_res$	*k, N_res*	*Geeraerd et al., 2000*
Log linear + shoulder + tail	$N= (N_0- N\_res) * exp(-k * t) * ((exp(k * Sl)))/(1+(exp(k * Sl) - 1) *exp(-k*t))) + N\_res$	*k, N_res, Sl*	*Geeraerd et al., 2000*
Weibull	$N/N_0= 10^{\wedge} (-((t/a) ^{\wedge}n))$	*a, n*	*Mafart et al., 2002*
Biphasic model	$N=N_0 (f*exp(-k_1*t)+(1-f)*exp(-k_2*t)$	f, k_1, k_2	*Cerf, 1977*
Biphasic + shoulder	$log_{10}(N)=log_{10}(N_0)+log_{10}(f*exp(-k_1*t)*(exp(k_1*Sl))/(1+(exp(k_1*Sl)-1)*exp(-k_1*t))+(1-f)*exp(-k_2*t)*(exp(k_2*Sl))/(1+(exp(k_1*Sl)-1)*exp(-k_1*t))^{\wedge}(k_2/k_1))$	f, k_1, k_2, Sl	*Geeraerd et al., 2006a & 2006b*

Results and discussion

Preliminary tests

In the first instance, a preliminary test was carried out to select the best DiaCell configuration. The experiment used fresh-cut produce process water as described in material and methods. Results indicated that DiaCell 101 produced free chlorine (data not shown) and disinfection of TW containing organic matter faster than DiaCell 401 (Figure 1). The faster production of free chlorine by DiaCell 101 can be caused by the higher total current/higher current densities applied (see materials and methods). This fact is in concordance with that reported by other studies which state that a higher rate of production of oxidants has been reported at higher current densities (Kraft et al., 1999; Scialdone et al., 2009; Schmalz et al., 2009). In contrast, it was also reported that an increase in current density does not imply an increase in oxidation and disinfection efficiency (Anglada et al., 2009; Schmalz et al., 2009). In our study, if the level of disinfection is plotted against the electrolyzed charge instead of the time (data not shown) the efficiency of both cell configurations is similar (e.g. 0.7 A·h/L to obtain a 4 log reductions). Under the specified conditions, the power requirement of DiaCell 101 is higher than those of Diacell 401 (500 W and 25 W, respectively) and therefore, Diacell 101 supplied a higher amount of heat to the water. Consequently, DiaCell 401 was selected for further experiments due to its potential use in the fresh-cut industry where refrigerated water is required for vegetable washing.

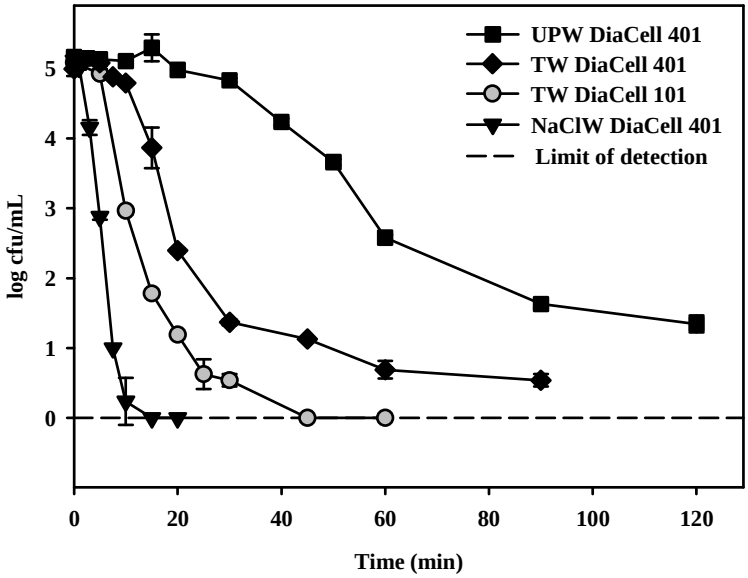


Figure 1. Evolution of the level of *E. coli* O157:H7 in different model waters during treatment with the DiaCell technology. Model waters treated were: Ultra pure water (UPW) supplemented with Na_2SO_4 and $NaHCO_3$, tap water (TW), and tap water supplemented with NaCl (NaClW). All the model waters had a COD of 800 mg/L. The values are shown as symbols connected by a solid line and vertical bars represent the standard deviation.

Once the most suitable cell was selected (DialCell 401), the efficacy in reducing NalR *E. coli* O157:H7 was evaluated in UPW (i.e. in absence of chloride). Results showed that the levels of *E. coli* O157:H7 were reduced 2.5 log units after 60 min and 3.8 log units after 120 min (Figure 1). Likewise, COD was reduced by almost 50 % (from 800 to 410 mg/L) at the end of the treatment (120 min). As expected, free chlorine levels were always below the limit of detection (0.03 mg/L) during the treatment. As water utilised for this experiment contained no chloride, the total chlorine detected (1.15 mg/L

after 120 min) is an interference of the analysis method that could be derived from the hydrogen peroxide produced as a consequence of the electrolysis of water. This hypothesis can be confirmed as a level of around 10 mg/L of H_2O_2 was detected at the end of the treatment. Therefore, it could be proposed that disinfection and oxidation of organic compounds, in this treatment, could have been result of the direct oxidation at the anode surface, but also of the electrochemical production of non-chlorine-based oxidants (H_2O_2,$S_2O_8^{2-}$, $C_2O_6^{2-}$, and ROS) in the applied conditions as previously reported (Jeong at al., 2009). Results obtained here confirm the well-known potential of the electrochemical treatment with BDD electrodes for disinfection and reduction of COD of water even in chloride-free waters (Martínez-Huitle & Ferro, 2006; Martínez-Huitle & Brillas, 2008; Scialdone et al., 2009). Nevertheless, there is scientific evidence proving that disinfection and oxidation of organic matter is faster when using chloride-containing waters (Kerwick et al., 2005; Martínez-Huitle & Ferro, 2006; Schmalz et al., 2009). Hence, in our preliminary study, disinfection efficiency of UPW with DiaCell 401 was low compared with that of the treatments of TW and much lower than the treatment of NaClW (Figure 1). For example, after 120 min of treatment, around 1.2 log cfu/mL of inoculated bacteria could still be detected in UPW, while similar levels of survival bacteria were detected in TW and NaClW after shorter treatment times (30 and 7.5 min respectively). Considering application in the fresh-cut industry, only TW and NaClW were selected for further experiments.

Tap water (TW) treatment with DiaCell 401

In this study, great differences were observed in the contact time required to reduce *E. coli* O157:H7 from 10^5cfu/mL to the detection limit among different COD levels (Table 4). As an example, levels of *E. coli* O157:H7 were below the limit of detection (1 cfu/mL) after 0.9, 25 min, and 60 min for TW with a COD of 60, 300 and 600 mg/L, respectively (Table 4). In a previous study, Ongeng et al. (2006) evaluated the efficacy of electrochemical disinfection of fresh-cut produce process water. In their study and using spoilage bacteria, they found that faster and larger microbial load reductions could be achieved in process water with lower organic load. This is in concordance with our results using *Escherichia coli* O157:H7 strains.

For TW with a COD of 800 mg/L, levels were below the limit of detection after 90 min in one of the three repetitions, and below 1 log in the other two repetitions. Consequently, concentrations of 10^5cfu/mL required about 0.02, 0.53, 1.28 and 1.92 A·h/L electrical charges, respectively, to bring down the level 5 log units in TW with a COD of 60, 300, 600 and 800 mg/L, respectively (Table 4).

Regarding chlorine and ORP levels, in the treated TW with a COD value of 60 mg/L, chlorine produced by the cell remained as free chlorine from the beginning, reaching a level of 0.85 mg/L after 1.5 min, while ORP remained around 400 mV (Figure 2A). In contrast, free chlorine levels remained low and chlorine was almost completely in a combined form until the end of the treatment in TW with 300, 600 and 800 mg/L of COD, respectively (Figures 2B, 2C & 2D). ORP sharply decreased just after starting the treatment, slightly increased during the treatment, and remained in the range of 150-300 mV until the end of the treatment (Figures 2B, 2C & 2C).

Table 4. Time (min) and electrical charge (A·h/L) needed to reduce the levels of *E. coli* O157:H7 from 10^5cfu/mL to the limit of detection (1 cfu/mL) in tap water (TW) and tap water supplemented with NaCl (NaClW) with different levels of chemical oxygen demand (COD) treating with DiaCell 401.

Model water	COD (mg/L)	Time (min)	Charge (A·h/L)
TW	60	0.9	0.02
	300	25	0.53
	600	60	1.28
	800	90	1.92
NaClW	60	0.17	0.003
	300	4	0.08
	500	15	0.32
	750	24	0.51

Interestingly, during bacterial inactivation, independently of the COD level (except in the case of TW COD 60 mg/L), the inactivation started when level of total chlorine was in the range of 2-3 mg/L (Figure 2). Furthermore, average levels at the end of the treatment of total chlorine, combined chlorine and free chlorine, were close to 8 mg/L, 8 mg/L, and 0 mg/L, respectively, whereas ORP was around 200 mV (Figure 2). Regarding organic matter oxidation, it was observed that COD decreased by 13.0±3.7 %, 32.8±0.8 %, and 47.9±11.3 %, after treatment times of 25, 60 and 90 min, respectively in TW with an initial COD of 300, 600 and 800 mg/L. Taking into account the length of the treatment time, the percentage of COD reduction/min was similar (0.53±0.01), independently of the initial COD of the water. These results are in broad agreement with a mechanism of direct COD oxidation at the anode surface (Anglada et al., 2009).

Tap water supplemented with NaCl (NaClW) treatment with DiaCell 401

As expected, shorter times and therefore, lower electrical charges were sufficient to decrease the levels of inoculated bacteria (10^5cfu/mL) below the limit of detection in NaClW as compared to TW (Table 4). Other authors have reported an increase in the efficacy of this technology when chloride ions are added (Kerwick et al., 2005; Martínez-Huitle& Ferro, 2006; Vijayaraghavan et al., 2006; Schmalz et al., 2009; Mascia et al., 2010). As in the case of TW treatment, COD affected the efficacy of NaCl water treatment on the inactivation of *E. coli* O157:H7. Levels of *E. coli* O157:H7 were below the limit of detection (1 cfu/mL) after 0.17, 4 and 15 min for NaClW with a COD of 60, 300 and 500 mg/L, respectively, while in the case of NaClW with a COD of 750 mg/L (maximum level assayed for NaClW), concentration levels were below the limit of detection after 25 min in two of the three repetitions of the experiment (Table 4).

In the NaClW treatment with a COD of 60 mg/L, from the start of the treatment, chlorine produced by the cell remained as free chlorine and ORP increased (Figure 3). Free chlorine levels remained low and chlorine was almost completely in a combined

form for 1, 5 and 10 min in NaClW with 300, 500 and 750 mg/L of COD, respectively (Figure 3). Evolution of the proportions of the different types of chlorine is explained by the differences in COD concentrations. In these three experiments, ORP increased from the start of the treatment, but the increase became faster when free chlorine levels started to rise. Independently of the COD level (except in the case of NaClW COD 60 mg/L), average levels of total chlorine, free chlorine and combined chlorine at the end of the treatments were close to 30 mg/L, 20 mg/L, 10 mg/L respectively, and the ORP value was above 600 mV. It is important to point out that the levels of free chlorine measured in NaClW with 300, 500 and 750 mg/L would be sufficient to inactivate rapidly the bacteria present in clean water. In contrast, in our experiments cultivable *E. coli* O157:H7 could be found during few minutes in the water in presence of free chlorine. Possible explanations could be the competition of the organic matter with the bacteria as a target for the free chlorine, the attachment of the bacteria to organic particles, and the stabilization of bacterial structures by the organic matter present in the water (Virto et al., 2005).

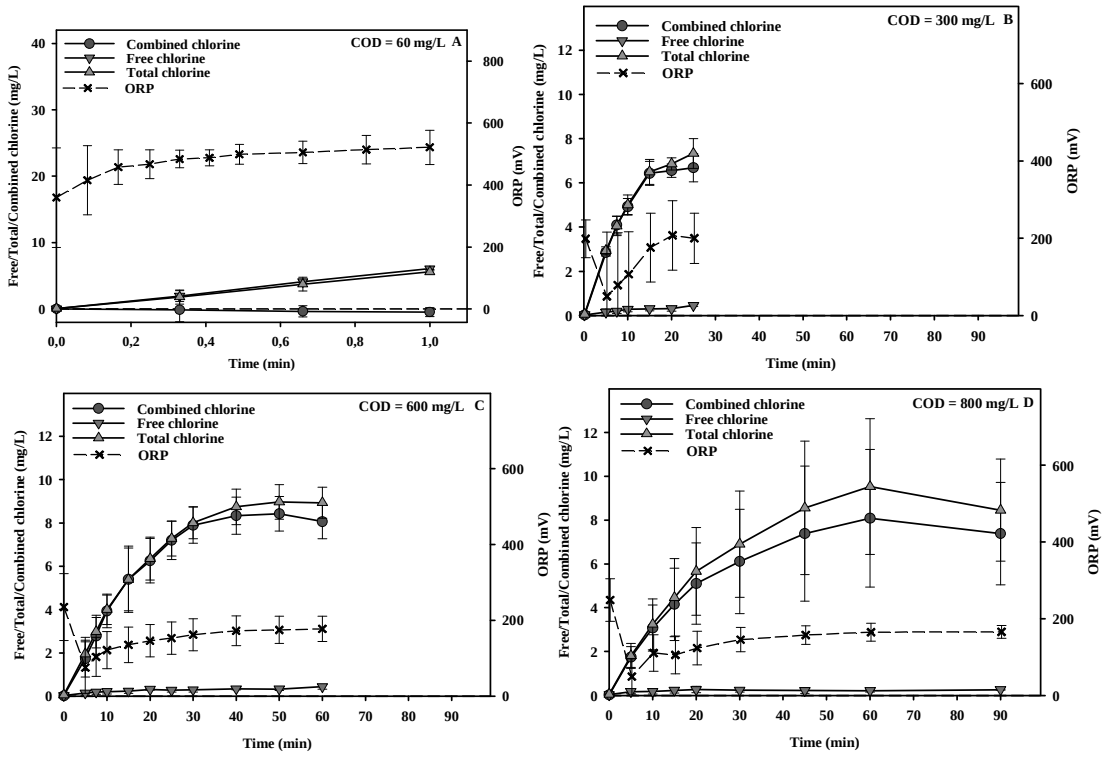
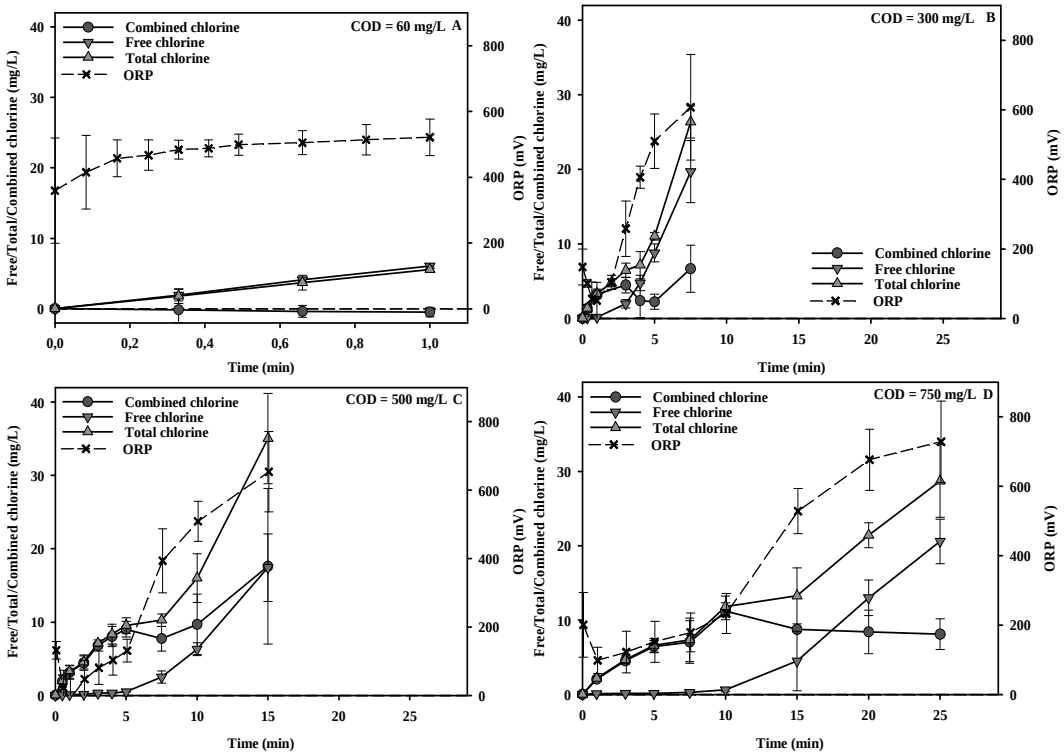


Figure 2. Evolution of the levels of chlorine (mg/L) and ORP (mV) during treatment of tap water (TW) with different chemical oxygen demands with DiaCell 401. Values are the mean of three replicates and vertical bars represent the standard deviation.

Regarding organic matter oxidation, it was observed that COD decreased by 15.9±10.0 %, 14.3±8.2 %, and 11.7±7.3 %, in NaClW with a COD of 300, 500 and 750 mg/L, after 7.5, 15 and 25 min, respectively, at the end of the treatment time. In contrast with the TW treatment, in the NaClW treatment the COD reduction rate was higher at lower COD of the water (% reduction/min: 2.1, 0.9 and 0.5 for a COD of 300, 500 and 750, respectively). These results suggest that the COD reduction would be caused by indirect oxidation by free chlorine, whose effectiveness was affected by the amount of organic matter. In processing plant conditions, the amount of chlorine present in the water could be used as a control parameter of the treatment efficacy and depending on chlorine levels, flow rate and current density could be managed in order to optimize the process.



Figures 3. Evolution of the levels of chlorine (mg/L) and ORP (mV) during treatment by DiaCell 401 of tap water supplemented with NaCl (NaClW) with different chemical oxygen demands. Values are the mean of three replicates and vertical bars represent the standard deviation.

Disinfection kinetic curves

The disinfection kinetic curves obtained for *E. coli* O157:H7 in both, TW and NaClW, exhibited three phases: shoulder, exponential decline, and tail (Figures 4 and 5). The initial phase showed a shoulder because of there was not a significant population decrease. Next, *E. coli* O157:H7 population experienced a rapid decline which was characterized, in the curve, by a steep slope. Finally, the exponential decay slowed down to result in a tailing effect in the curve. The shoulder can be explained by a single-hit multiple-target or a series event phenomenon in which the damage to the cell is accumulative rather than instantly lethal. This concept has been suggested to account for bacterial thermal inactivation (Geeraerd et al., 2000) and for bacterial inactivation under high energy UV-C irradiation (Labas et al., 2005) and ozone (Selma et al., 2006; 2007). Also, the presence of shoulder could be explained by the competitive consumption of oxidants by organic compounds present in the water as suggested by Schmalz et al. (2009). Consequently, the shoulder should be longer when higher amounts of organic matter are present in the water.

In our study, inactivation curves in NaClW have shorter shoulders and higher slopes than in TW (Figures 4 and 5). On the other hand, in both water samples (TW and NaClW) shoulders were shorter than for higher COD levels when the bacterial disinfection was carried out with a COD= 60 mg/L. However, in TW, for COD values of 300, 600 and 800 mg/L the length of the shoulders was similar (Figure 4). Therefore, the contact times and electrical charges of the treatments for obtaining the first log reduction (1D) of *E. coli* O157:H7 were similar for TW with 300, 600 and 800 mg/L of COD as shown in Figure 6A. This finding means there was an effect of COD on disinfection efficacy in TW but it was not linear. Unlike the TW, in NaClW the shoulder

was much longer with a maximum COD (i.e. 750 mg/L) than with lower COD values (i.e, ≤ 500 mg/L). Therefore, the contact time and electrical charge required to reach 1 D reduction in NaClW was higher with a COD of 750 mg/L than with 60, 300 or 500 mg/L (Figure 6B).

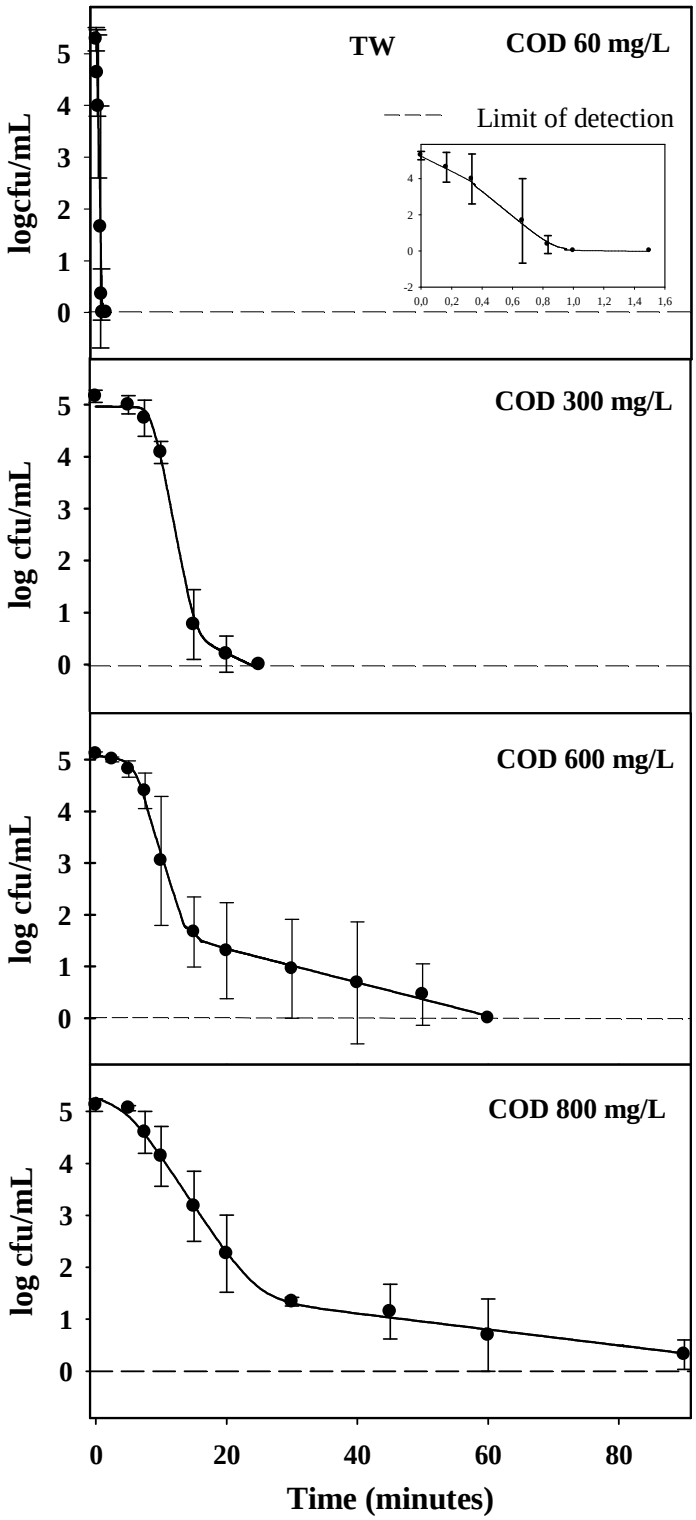


Figure 4. Evolution of the level of *E. coli* O157:H7 in tap water (TW) with different chemical oxygen demands during treatment with DiaCell 401.The mean of the three replicates is shown as symbols and vertical bars represent the standard deviation. Values obtained with the selected model are represented by a line. Small graph inserted shows in detail the inactivation of water with a COD of 60 mg/L.

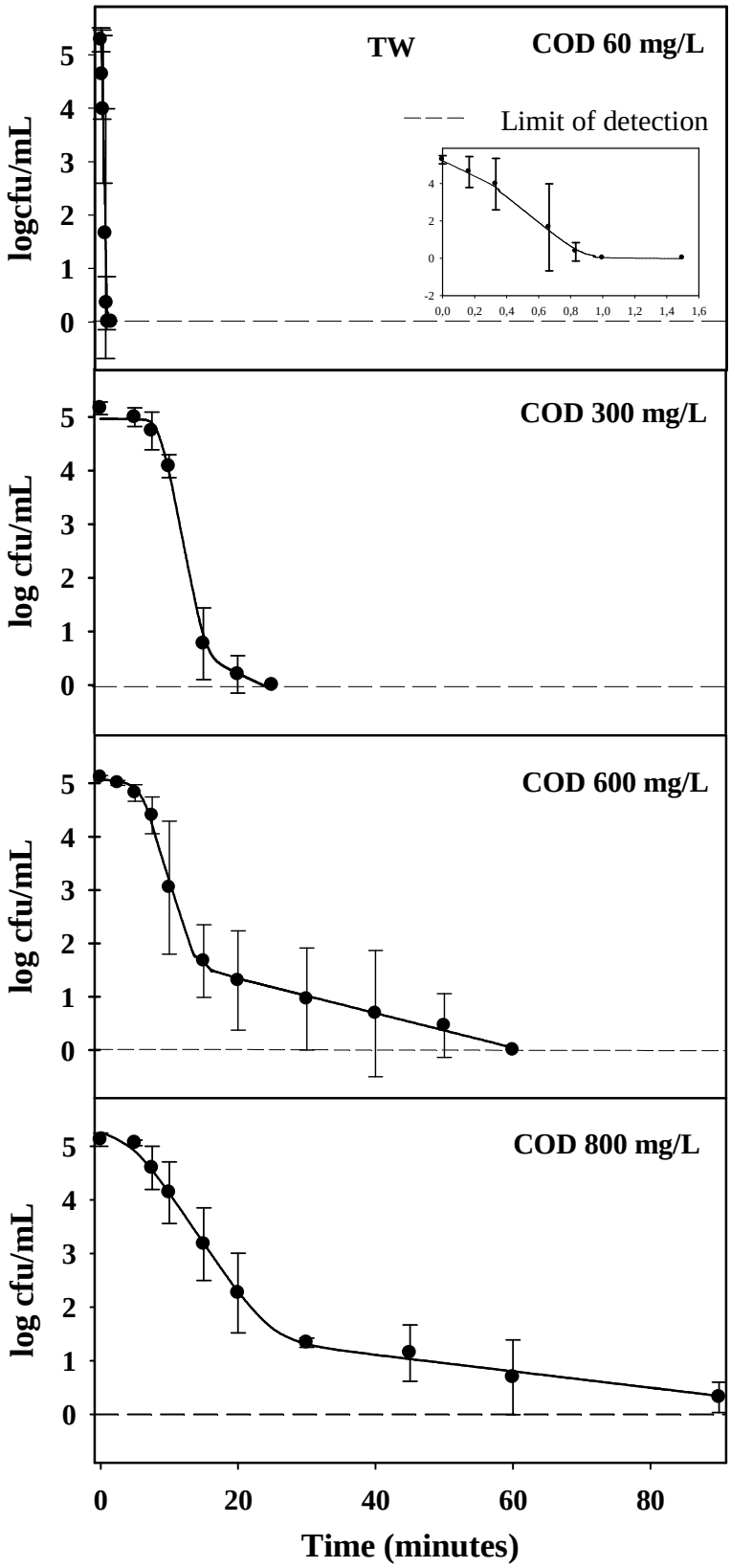


Figure 5. Evolution of the level of *E. coli* O157:H7 in tap water supplemented with NaCl (NaClW) with different chemical oxygen demands during treatment with DiaCell 401.The mean of the three replicates is shown as symbols and vertical bars represent the standard deviation. Values obtained with the selected model are represented by a line. Small graph inserted shows in detail the inactivation of water with a COD of 60 mg/L.

This difference in the shoulder length at different COD levels, with respect to the TW treatment, could be caused by the lower contribution to the overall disinfection rate of direct oxidation at the anode surface and/or indirect oxidation by oxidants different to chlorine in NaClW treatment, where chlorine is the main active disinfectant. Thus, in TW treatment, anodic oxidation and oxidation by certain oxidants different from chlorine would be less affected by the presence of organic matter than the oxidation based on the action of free chlorine. The exponential and tail phases were strongly affected by the amount of organic matter in TW and NaClW. Hence, the observed slopes were different depending on the COD of the water (Figures 4 and 5). Notably, the specific rates were lower at higher COD. Therefore, the consumption of oxidants by organic compounds of the water affects the disinfection rate. Consequently, the contact time and electrical charge of the treatment for achieving 4 log reductions (4D) was higher at higher COD (Figure 6). Tails are commonly detected when survival curves go beyond 4–5 log cycles (Smelt et al. 2002). Taking into account that the treatments were carried out in water in which well-mixed conditions assure that all the bacteria should receive the same lethal dose, the tail seems to be related to an inhibition phenomenon produced by the competition of the organic products released to the medium, as suggested by Benabbou et al. (2007) for photocatalytic disinfection.

Data Modeling

Non-linear regression analyses were used to study the experimental reductions of *E. coli* O157:H7 in TW with different COD. In a preliminary study, we used seven inactivation models which are included in Table 3. Disinfection process did not follow a log-linear kinetic although most of models presented good fitting to data (i.e. high R^2 and low RMSE) (Tables 5 and 6). Overall, the kinetic modeling of disinfection processes of NaClW treatment was similar to that of TW treatment. Only at very low COD (60 mg/L), no shoulder was found in NaClW treatment (Figure 5). This could be the result of a very rapid generation of free chlorine in water which caused effective disinfection to start earlier. The models which best fitted the experimental results were the Weibull model and bi-exponential model (biphasic) due to their similarity to the decreasing pattern shown by *E. coli* O157:H7 and the higher values obtained for the goodness-of-fit statistics (Tables 5 and 6). The biphasic model showed R^2>0.86 for all assayed conditions. When the model was extended to include an initial shoulder (see Figures 4 and 5), fitting improved noticeably, R^2 reaching values above 0.98 excepting for treatment with NaClW at COD= 60 mg/L in which R^2 was equal to 0.50 as a consequence of the inactivation curve that did not present shoulder (Tables 5 and 6).

Biphasic model has been proposed as alternative to the typical log-linear model to explain inactivation curves have noticeable upward concavity (Peleg, 2006). This model is proof that there is a mixture of two or more subpopulations with different inactivation rates. In our study, inactivation curves clearly showed two disinfection rates which could correspond with subpopulations with different resistance levels to the disinfection treatment. Model parameters confirmed numerically such a difference, and indicated that first disinfection rate (k_1) decreased significantly when COD increased from 60 to 300 mg/L, for treatments in TW and NaClW, this increase being less evident at higher COD (> 300 mg/L) (Tables 7 and 8). The second disinfection rate (k_2) was lower than k1 in all cases, being < 0.3 for conditions assayed in NaClW (Table 8). This result can be confirmed visually in the inactivation curves presented in Figure 5 which showed a tailing effect with a reduced or non existing slope as observed for COD= 60 mg/L (Table 8).

Table 5.Comparison of RMSE.R^2 and adjusted R^2 for the proposed models on the inactivation of *E. coli* by electrochemical treatment in tap water (TW).

COD (mg/L)		Log linear	Log linear + shoulder	Log linear + tail	Log linear + shoulder + tail	Weibull	Biphasic model	Biphasic + shoulder
60	RMSE	11.444	1.0972*	0.2514	0.1808	0.9090	0.4411	0.4230
	R^2	0.7519	0.8045	0.9897	0.9956	0.8658	0.9690	0.9806
	adjusted R^2	0.7164	0.7393	0.9863	0.9929	0.8211	0.9558	0.9613
300	RMSE	0.8994	0.8916	0.9304	0.1706	0.9781	0.8969	0.2327
	R^2	0.8827	0.9078	0.8996	0.9975	0.8890	0.8686	0.9969
	adjusted R^2	0.8592	0.8617	0.8494	0.9949	0.8335	0.8029	0.9906
600	RMSE	0.9449	0.8888*	0.4276	0.3834	0.7375	0.3757	0.1135
	R^2	0.8021	0.8443	0.9640	0.9747	0.8928	0.9757	0.9981
	adjusted R^2	0.7801	0.8054	0.9550	0.9638	0.8660	0.9652	0.9968
800	RMSE	0.9024	0.8389*	0.3232	0.3009	0.6739	0.2649	0.1174
	R^2	0.7926	0.8431	0.9767	0.9827	0.8988	0.9866	0.9978
	adjusted R^2	0.7666	0.7983	0.9701	0.9741	0.8699	0.9799	0.9960

RMSE: Root mean sum of squared errors. R^2: coefficient of determination.
*model unlikely for this data

Table 6. Comparison of RMSE, R^2 and adjusted R^2 for the proposed models on the inactivation of *E. coli* by electrochemical treatment in tap water supplemented with NaCl (NaClW).

COD (mg/L)		Log linear	Log linear + shoulder	Log linear + tail	Log linear + shoulder + tail	Weibull	Biphasic model	Biphasic + shoulder
60	RMSE	11.770	0.4639*	0.2684	0.0128*	0.5447	0.6233	12.965
	R^2	0.7118	0.9664	0.9888	0.9999	0.8877	0.8693	0.5052
	adjusted R^2	0.6397	0.9440	0.9813	0.9999	0.8627	0.8203	0.2225
300	RMSE	12.657	1.2813*	0.6021	0.0987	11.183	0.7539	0.6439
	R^2	0.7623	0.7835	0.9522	0.9989	0.8350	0.9344	0.9590
	adjusted R^2	0.7359	0.7293	0.9402	0.9984	0.7938	0.9063	0.9317
500	RMSE	10.839	1.0063*	0.3503	0.3352	0.7903	0.4183	0.4595
	R^2	0.7689	0.8229	0.9785	0.9828	0.8908	0.9732	0.9723
	adjusted R^2	0.7432	0.7786	0.9732	0.9754	0.8635	0.9617	0.9539
750	RMSE	0.5615	0.5800*	0.3346	0.3307	0.5033	0.3148	0.2191
	R^2	0.9324	0.9382	0.9794	0.9833	0.9535	0.9841	0.9936
	adjusted R^2	0.9228	0.9176	0.9726	0.9732	0.9379	0.9761	0.9884

RMSE: Root mean sum of squared errors. R^2: coefficient of determination.
*model unlikely for this data

Table 7. Model parameters and Standard Error estimated by non-regression analysis for inactivation of *E. coli* O157 by electrochemical treatment in tap water (TW).

Model Name		60 (mg/L)*		300 (mg/L)		600 (mg/L)		800 (mg/L)	
	Model parameters	Value	S. E.	Value	S. E.	Value	S. E.	Value	S. E.
Weibull	a	0.0528	0.069	5.1547	3.5920	1.8658	2.0320	2.9656	3.0052
	n	0.5185	0.170	1.1712	0.4779	0.5191	0.1458	0.5209	0.1400
Biphasic model	f	0.9999	0.0001	Nf	Nf	0.9999	0.0002	0.9993	0.0002
	k_1	13.7839	1.9974	Nf	Nf	0.5527	0.0780	0.3476	0.0380
	k_2	2.5932	1.3857	Nf	Nf	0.0606	0.0325	0.0347	0.0164
Biphasic + shoulder	f	0.9999	0.0001	0.9999	0.0140	0.9995	0.0019	0.9997	0.0002
	k_1	13.5725	3.0478	1.5149	33.7886	1.0494	0.5005	0.4460	0.0429
	k_2	1.5430	1.4712	0.1373	0.0759	0.0747	0.0062	0.0392	0.0060
	Sl	0.0462	0.1249	8.5602	57.6346	5.7772	0.9108	4.1493	1.1273

*Chemical Oxygen Demand (COD); S.E.: Standard Error; a: The scale parameter; n: the shape parameter; f: the fraction of the initial population in a major less-resistant subpopulation; $k_1$and k_2: the specific inactivation rates of the two subpopulations; Sl: Shoulder length; N.f.: Data cannot be modeled.

Table 8. Model parameters and Standard Error estimated by non-regression analysis for inactivation of *E. coli* O157 by electrochemical treatment in tap water supplemented with NaCl (NaClW).

Model Name		60 (mg/L) *		300 (mg/L)		500 (mg/L)		750 (mg/L)	
	Model parameters	Value	S. E.	Value	S. E.	Value	S. E.	Value	S. E.
Weibull	a	0.0001	0.0006	0.2747	0.4180	0.4273	0.4964	2.2924	1.2726
	n	0.1600	0.0735	0.5443	0.2135	0.4927	0.1360	0.7348	0.1578
Biphasic model	f	0.9996	0.0006	0.9999	0.0003	0.9999	0.0001	0.9997	0.0008
	k_1	30.3472	9.7321	2.9465	0.7218	1.4252	0.1968	0.7358	0.0832
	k_2	0.0000	2.4046	0.4244	0.5376	0.1397	0.1286	0.1537	0.1474
Biphasic + shoulder	f	1.0000	0.0001	1.0000	0.0002	1.0000	0.0001	0.9960	0.0207
	k_1	30.3463	19.0420	3.5634	2.4305	1.6406	0.5393	1.4003	1.2195
	k_2	0.0000	6.4306	0.1514	0.3548	0.0938	0.1213	0.2899	0.0390
	*Sl***	0.0000	0.1739	1.0022	0.9422	1.1877	1.1575	3.4473	1.2131

*Chemical Oxygen Demand (COD); S.E.: Standard Error; a: The scale parameter; n: the shape parameter; f: the fraction of the initial population in a major less-resistant subpopulation; k_1 and k_2: the specific inactivation rates of the two subpopulations; Sl: Shoulder length.

In contrast, for treatment in TW, k2 presented a higher value for COD= 60 mg/L (1.540), although this decreased to values ≤0.080 for COD≥300 mg/L (Table 7). Note that parameter f is the relative weight fraction of each disinfection rate (i.e. k_1 and k_2) in the mixed population. This parameter (f) was much lower for second disinfection phase in all cases, since less sensitive *E. coli* O157:H7 subpopulation to the disinfection treatment was much smaller. In all cases, the parameter accounting for shoulder length (Sl) increased as COD increased as already commented in previous sections.

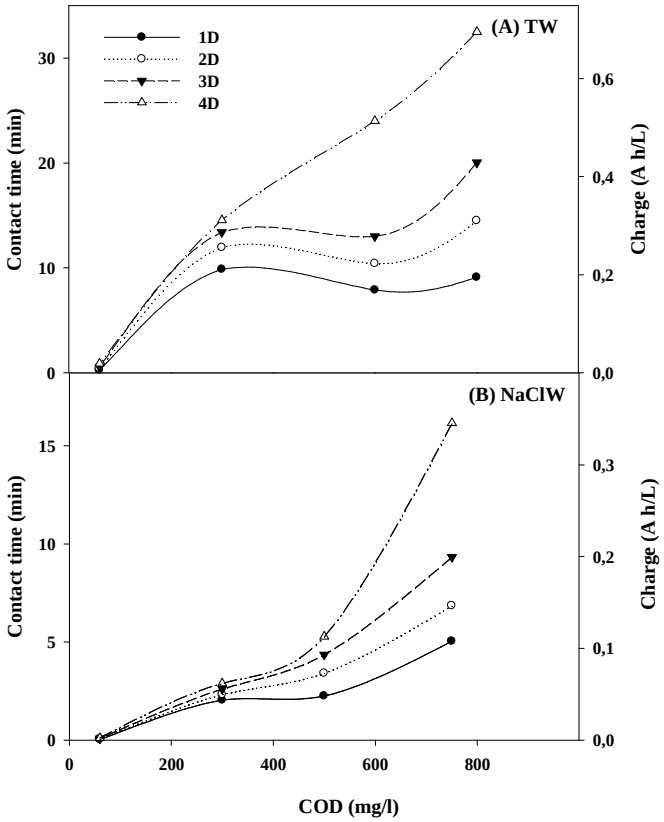


Figure 6. Contact time (min) and electrical charge (A·h/L) needed to achieve D-log reductions of *E. coli* O157:H7 in tap water (A) and tap water supplemented with NaCl (B) with different COD levels.

The Weibull model presented lower R^2 (<0.86) than those shown by the biphasic + shoulder model. This type of model has been widely applied to describe inactivation by heat treatment at isothermal conditions; although due to its flexibility, it has also been successfully used to model inactivation by exposure to chemical agents or other disinfection technologies (Corradini & Peleg, 2003). The Weibull model is based on the fact that microbial inactivation by physical or chemical inactivation treatment can be viewed as a failure phenomenon: failure of microorganisms to resist the harsh conditions (Peleg, 2006). The Weibull parameter *a* is considered as a reaction rate constant and *n* as a behavior index. This model reduces to a linear model when *n* =1, however no case in our study presented *n* =1, which means the disinfection process did not follow a log-linear kinetic (see Tables 7 and 8).

Furthermore, when *n*> 1, the inactivation curve shows downward concavity which indicates that remaining cells become increasingly damaged; while when n< 1, the inactivation curve shows upward concavity which indicates that remaining cells have

the ability to adapt to applied stress (van Boekel, 2002). The Weibull model fitted to inactivation curves of *E. coli* O157:H7 presented $n < 0.5$ with exception of the treatment in TW at COD=300 mg/L in which $n = 1.170$ although this condition also resulted in a high Standard Error (SE) as compared to other conditions (see Tables 7 and 8). Although *n* is dependent on environmental factors (e.g. COD), some authors have considered *n* constant, assigning it a fixed value for fitting purposes without effecting significantly goodness of fit of the model. In our study, *n* seemed to be similar for the range COD=300-800 mg/L in both water types, TW and NaClW (n= 0.490-0.520) with the exception of the aforementioned case (i.e., TW at COD=300 mg/L). Therefore, this fact suggests that *E. coli* O157:H7 at high COD levels (≥300 mg/L) in electrolyzed water (with BDD electrodes) exhibits a similar disinfection pattern i.e. a characteristic upward concavity-pattern. On the other hand, parameter *a* increased as COD increased for NaClW which means, according to the Weibull function, that disinfection rate decreases when COD increases (Table 8). In TW, this fact was only evident between COD =60 and 300 mg/L where *a* increased from 0.053 to 5.150, respectively. Therefore, both models (Biphasic+shoulder and Weibull) could successfully account for the observed inactivation kinetic of *E. coli* O157:H7 in electrolyzed TW and NaClW, reflecting the disinfection rate reduction caused by increasing COD levels and the existence of a survivor subpopulation of the *E. coli* O157:H7, less sensitive to electrolyzed water.

Conclusions

In this study, electrochemical treatment with BDD electrodes showed potential for disinfection and for reducing COD of water containing lettuce organic matter. From the applicability point of view, this technology could be used in the fresh-cut industry to maintain the microbiological and physicochemical quality of wash water, reducing chlorination by-products formation with respect to sodium hypochlorite use. Electrochemical treatment could be applied after the vegetable washing, to improve the water quality in order to allow its reuse or to re-condition the wastewater before discharge. For these uses, it would be possible to treat the water electrochemically without addition of chloride, minimizing the formation of chlorination by-products. It could also be applied for re-conditioning the water immediately before the vegetable washing, improving quality of the water and generating microbicidal species that would provide the necessary residual disinfectant power in the washing tanks. For this use, based on the information obtained during this study, addition of chloride would be unavoidable. Disinfection kinetic models need to be determined for predicting the influence of important parameters on the disinfection processes such as reaction time and organic demand. In our study, the biphasic+shoulder model and Weibull model were proposed as suitable models for accounting for the disinfection process of *E. coli* O157:H7 in electrolyzed water with BDD electrodes. In addition, basic research to identify the processes and factors that lead to the appearance of nonlinearity should be carried out.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7) under grant agreement no 244994 (project VEG-i-TRADE, www.veg-i-trade.org) and the MICINN (projects AGL2010-20070 and AGL2009-08603). Special thanks to the Company Adamant Technologies SA (La Chaux-de-Fonds, Switzerland) for providing us with the technology and support

required to complete the project. M.V. Selma is holder of a Ramón y Cajal contract, G.D. Posada-Izquierdo is holder of a predoctoral scholarship and F. López-Gálvez of a postdoctoral scholarship from the MCINN.

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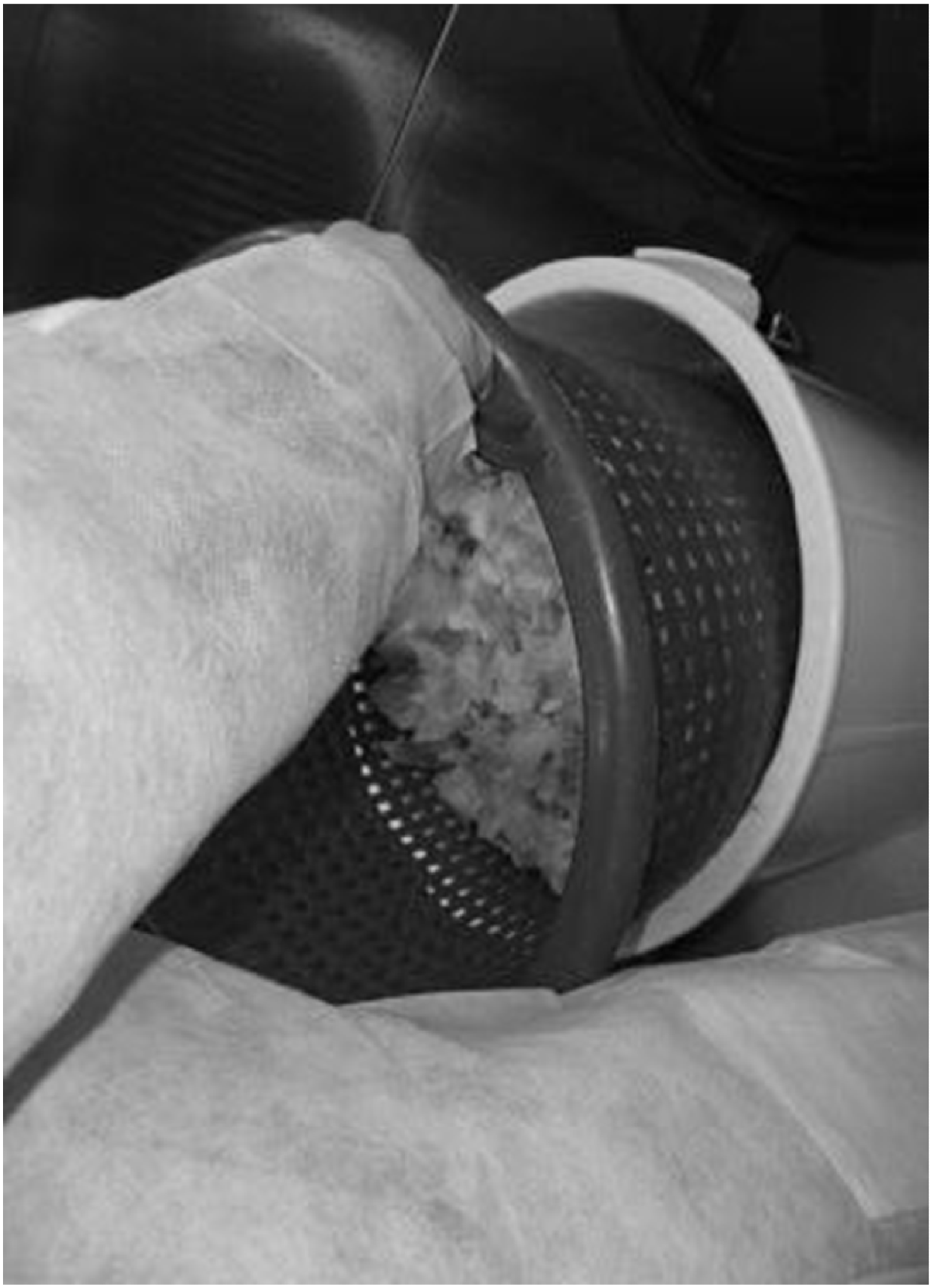

Chapter III: "Modelling growth of *Escherichia coli* O157:H7 in fresh-cut lettuce submitted to commercial process conditions: chlorine washing and modified atmosphere packaging"

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Published in: Food Microbiology 33, 131-138, 2013

Abstract

Fresh-cut iceberg lettuce inoculated with E. coli O157:H7 was submitted to chlorine washing (150 mg/mL) and modified atmosphere packaging on laboratory scale. Populations of *E. coli* O157:H7 were assessed in fresh-cut lettuce stored at 4, 8, 13 and 16 °C using 6-8 replicates in each analysis point in order to capture experimental variability. The pathogen was able to grow at temperatures ≥8 °C, although at lower temperatures, growth data presented a high variability between replicates. Indeed, at 8 °C after 15 days, some replicates did not show growth while other replicates did present an increase. A growth primary model was fitted to the raw growth data to estimate lag time and maximum growth rate. The prediction and confidence bands for the fitted growth models were estimated based on Monte-Carlo method. The estimated maximum growth rates (log cfu/day) corresponded to 0.14 (95 % CI: 0.06-0.31), 0.55 (95% CI: 0.17-1.20) and 1.43 (95% CI: 0.82-2.15) for 8, 13 and 16 ºC, respectively. A square-root secondary model was satisfactorily derived from the estimated growth rates (R2>0.80; Bf= 0.97; Af= 1.46). Predictive models and data obtained in this study are intended to improve quantitative risk assessment studies for *E. coli* O157:H7 in leafy green products.

Introduction

Over the last years, production and consumption of fresh-cut or minimally-processed (MP) fruit and vegetables has increased dramatically in many countries (Anonymous, 2007). However, MP vegetables pose serious risk since they do not undergo a process lethal to pathogenic bacteria (FSA, 2007; Carrasco et al., 2008; Pérez-Rodríguez et al., 2008; Gil et al., 2009).

Escherichia coli O157:H7 is a serious concern for the fresh-cut produce industry since vegetables may become contaminated in the field by contact with contaminated animal waste, dust, soil, irrigation water, and inadequately treated manure (Beuchat, 2002; EFSA, 2011; Pérez-Rodríguez et al., 2011). Epidemiological data suggest that the pathogen could transmit through the food chain, contaminating the final product (i.e. fresh-cut vegetables) and causing illness (USFDA, 2006). In the last three decades, many outbreaks of foodborne illness have been associated with the consumption of fresh and fresh-cut produce contaminated with *Escherichia coli* O157:H7 (Ackers et al., 1998; Rangel et al., 2005; Söderström et al., 2008).

Although knowledge about the transmission of the pathogen at different stages is scarce, it appears that cross-contamination at the factory, mainly during washing, could have a crucial role (Allende et al., 2008; Danyluk & schaffner, 2011; Doyle & Erickson, 2008; Gil et al., 2009). Disinfection of process wash water is a critical step in reducing cross-contamination; however, studies show that chlorinated water, the most used disinfectant

agent for the fresh-cut industry, as well as other commercial sanitizers, does not completely eliminate pathogens in produce (López-Gálvez et al., 2009; Zhang et al. 2009).

Studies have shown that *Escherichia coli* O157:H7 is able to survive during processing, and then recover and grow during refrigerated storage (Diaz & Hotchkiss, 1996; Delaquis et al., 2002; Oliveira et al., 2010). The exposure of bacteria to stress conditions like chlorine washing can affect their subsequent growth, depending on the intensity and duration of the stress. However, most studies investigating bacterial growth on MP vegetables have employed pathogenic bacteria cultured under non-stressful conditions (Abdul-Raouf et al., 1993; Bharathi et al., 2001; Koseki & Isobe, 2005; Luo et al. 2009; Oliveira et al. 2010). Moreover, few investigators have considered the effect of variables such as packaging conditions and chlorine washing on the growth of pathogens such as *E. coli* O157:H7 in MP vegetables (Lu et al., 2006; Lee & Baek, 2008).

Modified atmosphere packaging (MAP) can extend the shelf-life of cut lettuce primarily by providing a sufficiently low O_2 partial pressure (pO_2) to retard browning (Smyth et al., 1998). In this preservation technique, the air around the commodity is altered to a gas combination of 1 to 5 kPa pO_2 and 3 to 10 kPa pCO_2 (balance N_2) (Kader, 1980; Jacxsens et al., 1999). The applied packaging film is selected on the basis of O_2 and CO_2 transmission rates required to obtain a desirable equilibrium MAP inside the package (Jacxsens et al., 1999). The use of MAP is also intended to inhibit or retard the growth of spoilage and some pathogenic microorganisms, particularly due to the low O_2 concentration (Farber, 1991).

There are few published growth models for pathogenic bacteria in MP vegetables. In the study by Koseki & Isobe (2005), *E. coli* O157:H7 growth on non-packaged iceberg lettuce was successfully modelled by using the Baranyi model (Baranyi & Roberts, 1994), and then a secondary model based on the Ratkowsky model (Ratkowsky et al., 1982) was derived to predict growth parameters (maximum growth rate, latent phase and maximum density of population) as a function of temperature (5-25 °C). This study also highlighted that the predictions from broth-based models tend to overestimate growth potential in MP lettuce. More recently, Danyluk & Schaffner (2011) developed a secondary model for *E. coli* O157:H7 based on literature data dealing with *E. coli* O157:H7 growth in leafy vegetables. This work reported that broth-based growth models and also the model by Koseki & Isobe (2005) overestimate *E. coli* O157:H7 growth probably due to the model by Koseki & Isobe (2005) did not include MAP conditions. Also, McKellar & Delaquis (2011) have developed a secondary death-growth model based on data from different studies dealing with *E. coli* O157:H7 growth in leafy vegetables. This model permits predictions over a wide range of temperatures and also incorporates variability, thereby making it suitable for Quantitative Risk Assessment (QRA) studies. Predictive models that consider the influence of stresses such as washing in chlorinated water of MAP would provide more accurate and realistic estimates of growth and therefore of risk. Therefore, the aim of this study was to evaluate and model the effect of storage temperature on the growth of *E. coli* O157:H7 in fresh-cut lettuce subjected to chlorine washing and MAP.

Materials and methods

Bacterial strains and inoculum preparation

A five-strain cocktail of *Escherichia coli* O157:H7 strains isolated from human and

foods associated with hemorrhagic colitis and Haemolytic Uremic Syndrome (HSU), (ATCC 35150, CCUG 4076, ATCC 43894, ATCC 43895, and ATCC 11775) obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain) was used in the study. Cultures were rehydrated in Brain Heart Infusion broth (BHI, Oxoid, Basingtoke, United Kingdom). Nalidixic acid-resistant (Nal^R) *E. coli* O157:H7 cultures were obtained by consecutive 24 h transfers in BHI broth with increasing concentrations of nalidixic acid (Nal) (Merck, Darmstadt, Germany) until strains were resistant to 50 μg of Nal per mL BHI. Growth and survival experiments performed in different substrates or surfaces (BHI, lettuce, chard, spinach juices and on stainless steel surface) confirmed stability of the resistance of Nal^R *E. coli* O157:H7 strains and a similar behaviour to that shown by wild strains of *E. coli* O157:H7 (data not shown).

In order to guarantee a similar physiological status of the initial inoculum, Nal^R *E. coli* O157:H7 cultures were consecutively subcultured twice in 5 mL of BHI supplemented with 50 μg of Nal per mL (Nal^+) at 37 °C for 20 h. After the second incubation, equal volumes of the cultures were mixed. The resulting cell suspensions was washed three times by centrifugation (4100 *g*) at room temperature for 10 min with 0.1% $NaHCO_3$, and cells were suspended in 0.1% $NaHCO_3$ obtaining an inoculum with a level of 10^9 cfu/mL, approximately. Final concentrations of the inoculum solutions were confirmed by plating on Chromocult coliform agar (Merck, Barcelona, Spain) supplemented with 50 μg Nal per mL agar (Nal^+).

Preparation of vegetables and inoculation procedure

Iceberg lettuce (*Lactuca sativa L.*) was purchased from a local wholesale market in Murcia (Spain) at the day of harvest and transported to the laboratory within 1 h under refrigerated conditions. Lettuce was kept in darkness at 4 ºC overnight before the experiments. The three outer leaf layers, which usually correspond to dirty and damaged leaves, were manually removed and discarded while internal leaves were cut into 3x3 cm pieces (commercial size). Inoculation was performed by immersion of 14 Kg of the lettuce pieces in 10 L water containing $5x10^6$ cfu/mL of the inoculums cocktail for 1 minute. A level of inoculation of approximately 10^5 cfu/g lettuce was achieved using this procedure. Subsequently, the inoculated lettuce was dewatered by manual centrifugation with Paragourmet 90005 (Chef Master, Dominic Republic) applying ~ 50 rpm for 1 min and allowed to dry for 1 h at 4 ºC to favor adhesion of the *E. coli* O157:H7 cells to lettuce tissues, as previously suggested (Beuchat et al., 2003).

Sanitizing treatment and rinsing

The inoculated pieces´s lettuce were submitted to a standard chlorination treatment in cold tap water (4 ºC) containing 150 mg/L of total chlorine (NaClO) for 30 s. The sanitizing solution was prepared adding commercial sodium hypochlorite (NaClO, Lejıas Cortado S.L., Murcia, Spain) to 40 L of cold tap water (4 ºC). The pH of the sanitizing solution was adjusted to 6.5 using citric acid in order to improve chlorine disinfection efficacy. Fresh-cut iceberg lettuce was washed in different batches of 3.5 kg each during 30 s in the sanitizing solution at 4 ºC, followed by a rinsing step in tap water at 4 ºC. Finally, lettuce was centrifuged to remove excess water for 1 min with a manually-operated enclosed spinner (Paragourmet 90005). Changes in levels of free and total chlorine (mg/L), pH, and temperature (ºC), were measured in the washing solution before and after the disinfection of the vegetables. Temperature and pH were measured using a multimeter pH & Redox 26 (Crison, Barcelona, Spain). Free and total chlorine

were determined based on the N, N-diethyl-p-phenylendiamine (DPD) method (APHA, 1998) using the Spectroquant NOVA 60 photometer (Merck, Darmstadt, Germany). The processing was conducted under strict biosecurity conditions in an isolated processing room at 4 ºC. The involved personnel was trained in biosecurity practices, and during experiments, used adequate protective equipment (gloves, glasses, disposable head covering and mask) together with the application of a strict hand washing procedure. The processing room and equipment were disinfected before and after processing by applying a chlorine-based treatment.

Packaging and storage conditions

After processing, centrifuged lettuce was distributed in plastic bags and packaged under passive modified atmosphere conditions, in which the required atmosphere inside the bag is result of the vegetable respiration and the diffusion of gases through the film. The plastic bags were prepared using a coextruded film (Amcor Flexibles, Bristol, UK) with O_2 permeability of $9.365E^{-17}$ $molmm^{-2}$ s^{-1} Pa^{-1} (504 mLO_2 25µm/m^2 d atm) and CO_2 permeability of $3.166E^{-16}$ $molmm^{-2}$ s^{-1} Pa^{-1} (2507$mLCO_2$ 25µm/ m^2 d atm) at 7 ºC and 95% RH. A total of 124 bags of 12x12 cm containing 50 g of lettuce were prepared. The selected dimensions were chosen according to the ratio weight/volume used in commercial bags which contain 250 g with a dimension of 23x30 cm. Bags were divided in three different batches and each batch was stored under a specific temperature (40, 40 and 36 bags for 8, 13, 16 ºC, respectively). The remaining bags were stored at 4 ºC.

Sampling and microbiological analysis

A preliminary assay using lettuce leaves inoculated with Nal^R and wild strains *E. coli* O157:H7 and then exposed at different levels of chlorine (0-150 ppm) indicated a good recovery rate (> 36%) in Chromocult agar, Triptone Soy Agar (TSA) and Plate Count Agar (PCA) (Merck, Barcelona, Spain), finding no significant differences ($p>0.05$) between counts obtained for both types of strain and the different agars, particularly in an exposure time range of 10-60 s. Therefore, Chromocult agar was chosen to enumerate Nal^R *E. coli* O157:H7 in inoculated lettuce samples.

The population dynamics of Nal^R *E. coli* O157:H7 in the fresh-cut lettuce was measured at different time intervals distributed during storage with 6-8 replicates (i.e. bags) in each analysis point. The analysis times were defined for each temperature based on preliminary growth experiments (data not shown). For microbiological analysis, 50 g of lettuce (the total bag contents) were placed into a stomacher bag (Seward Limited, London, UK) containing 200 mL of 1 % buffered peptone water (Scharlau, Barcelona, Spain) and the mixture was homogenized for 2 min in a stomacher (IUL Instruments, Barcelona, Spain). The homogenate were diluted as required in 0.1 % buffered peptone water and were applied to the surface of Chromocult coliform agar supplemented with Nal^+ (50 µg/mL) using a spiral plater (model WASP, DW scientific, Shipley, UK). Plates were incubated at 37 ºC for 24 h before counting in an automated plate counter (ProtoCOL, Synoptics, Cambridge, UK).

Headspace analysis

Headspace gas composition (O_2 and CO_2) in individual packages were monitored using an O_2 analyzer with a ceramic oxide–zirconia electrochemical detection cell (CG-1000,

Ametek, Thermox Instruments Co., Pittsburgh, PA, USA) and an infrared CO_2 detector (Via 510, Horiba Instruments Co., Irvine, CA, USA). Two samples of 0.25 mL of headspace gas were removed from each package and measured.

Mathematical analysis

Primary model

Microbial population densities were log-transformed and introduced in Excel spreadsheet along with time (raw growth data). The primary growth model of Baranyi and Roberts (1994) was fitted to the raw growth data by means of DMFit, a Microsoft Excel Add-In developed by the Institute of Food Research, Norwich, UK, which calculated estimates of three kinetic parameters, i.e. latency time (lag, expressed in days in this paper), maximum growth rate (Grmax, expressed in log cfu/day) and maximum cell numbers (yend). A fitting method for repeated measures was applied to take into account results from the different replicates analyzed in each time interval.

Confidence intervals on the estimated kinetic parameters (Grmax and lag) as well as confidence and prediction bands for the fitted growth model were computed based on the Monte-Carlo method using the Standard Error (S_{yx}) reported by the DMFit program. The confidence intervals define the uncertainty range on the estimated regression parameters (Grmax and lag) while confidence bands define graphically the uncertainty range on the best-fit growth model (i.e. growth curve). Similarly, prediction bands represent the prediction error on the plotted best-fit growth model including both the uncertainty on the best-fit growth model and variability in data points.

To compute the confidence intervals, a normal distribution was defined with mean=0 and SD=S_{yx} which accounts for the range of error in the estimated concentration (log cfu/g) associated with the best-fit estimate for the model of Baranyi and Roberts (1994). Then, the normal distribution applied to each time point were simulated by using Monte-Carlo method with 1000 iterations for each one, resulting in a set of new 1000 growth curves. With the DMFit program, the Baranyi model was fitted to each new growth curve obtained by Monte-Carlo method, obtaining different estimates of the kinetic parameters for each growth curve. The 95 % confidence interval for each kinetic parameter was obtained based on 2.5^{th} and 97.5^{th} percentiles for each set of simulated kinetic parameter values. Similarly, the confidence bands (95 %) for the best-fit model were estimated based on 2.5^{th} and 97.5^{th} percentiles of the concentration predicted at each time by the growth models defined by the 1000 simulated kinetic parameters. The prediction bands were calculated based on the simulation data using the following equation (Montgomery et al., 2006):

$$Prediction\ width_i = \sqrt{(confidence\ width_i)^2 + (t_{\alpha/2,n-p} \times S_{yx})^2}$$

In the above equation, i is the index for growth time, S_{yx} is the Standard Error for the fitted model, $t_{\alpha/2,n-p}$ stands for the t-distribution with α being the probability (i.e. 0.05), n being the number of data points used to fit the model and p being the number of regression parameters. The *confidence width* corresponds to either the upper or the lower confidence bands, and the upper is estimated separately from the lower width

since the confidence bands from Monte-Carlo simulation does not have to be symmetric.

Secondary model and validation

Maximum growth rates (Grmax) estimated from Monte-Carlo method were used to derive a secondary model describing the relationship between growth rate and temperature. The applied secondary model corresponded to the square-root model described by Ratkowsky et al. (1982):

$$\sqrt{Gr\max} = \mathrm{b} \cdot (T - \mathrm{T}_{\min})$$

where b and T_{min} are regression parameters. The latter is considered the conceptual minimum temperature for microbial growth.

The model was fitted by least square linear regression with the software Statistica 10.1 (Statsoft Iberica, Lisbon, Portugal) and 95 % confidence bands were computed and plotted. The goodness of fitting was evaluated by applying the coefficient of determination (R^2) and Standard Error (SE). For validation purposes, model predictions were compared with growth data from literature by using Bias factor (B_f) and Accuracy factor (A*f*) described by Ross (1996), which can be calculated by using the following equations:

$$B_f = 10^{\left[\sum \log(gobs/gpred)/n\right]}$$

$$A_f = 10^{\left[\sum |\log(gobs/gpred)|/n\right]}$$

here, gobs and gpred stand for Grmax values taken from literature and Grmax values predicted by the model, respectively; n is the number of data.

Results

Behaviour of *Escherichia coli* O157:H7 in fresh-cut lettuce subjected to chlorine washing and MAP and stored at different temperatures.

The chlorination treatment followed by a rinsing step in tap water reduced the initial level of *E. coli* O157:H7 by 1.23 log cfu/g, from 4.66 to 3.43 log cfu/g, which is consistent with reduction values reported by other studies (Behrsing et al. 2000; Taormina & Beuchat, 1999). The obtained data indicated that surviving *E. coli* O157:H7 were able to grow at 8, 13, and 16 °C while at 4 °C, *E. coli* populations were reduced by 0.89 log cfu/g in 15 days. Regarding the gas composition inside the packages (initially equivalent to the atmospheric composition), the initial O_2 content decreased in the headspace of the washed fresh-cut lettuce during the storage reaching values lower than 0.1 kPa after 4 days of storage when product was stored at 16 ºC and after 6 days when fresh-cut lettuce was stored at 13 ºC (Table 1). In the case of 8 ºC, no measures were made in between 1-6 days, although at 6 days the level of O_2 presented values <0.1 kPa. On the other hand, the initial CO_2 content increased during storage up to 14 kPa approximately (e.g. after 3 days at 16 ºC) (Table 1). The generation of anaerobic atmosphere is associated with the biochemical activity of the vegetable tissue and native microflora growth but not with *E. coli* O157:H7 since anaerobic conditions occurred

without apparent *E. coli* O157:H7 growth in the analyzed lettuce as observed at 8 ºC for 6 days (Figure 1).

Pathogen populations increased by an average of 1.84 log cfu/g in samples stored at 8 ºC and reached an average maximum level of 5.27 log cfu/g (Figure 1). However, this increment only took place between days 15 and 27, approximately. Prior to this, there was a prolonged lag phase during which populations remained near inoculation levels. Interestingly, there was considerable variation between replicates in the experiment. Population increases of approximately 2 log cfu/g were measured in some samples after 15 days, but there was no evidence of growth in others. This large variability was also observed in the last analysis point (27 days) with differences of up to 2 log cfu/g between repetitions.

For samples stored at 13 ºC, a significant increase of *E. coli* O157:H7 was observed after three days of storage (Figure 2). After that, *E. coli* O157:H7 was able to reach an average maximum population density of 7.01 log cfu/g, which corresponds to an increase of 3.58 log cfu/g. This density was reached after 8 days, after which time populations began to decrease, reaching 5.89 log cfu/g by day 13. In parallel with observations at 8 ºC, there was considerable variability in outcomes between replicates. After 6 days in storage population densities differed by up to 2 log cfu/g.

Table 1. Gases composition (kPa) measured in inoculated lettuce bags over storage at different temperature

16 °C			**13 °C**			**8 °C**		
Days	pO_2	pCO_2	Days	pO_2	pCO_2	Days	pO_2	pCO_2
0	22.0±0.10*	0.33±0.10	0	22±0.10	0.33±0.10	0	22±0.10	0.33±0.10
1	0.41±0.04	10.97±1.72	3	0.25±0.15	12.40±0.97	6	0.09±0.10	14.27±7.81
2	0.21±0.20	12.84±1.76	6	0.07±0.11	11.35±0.92	10	0.43±0.53	10.44±1.22
3	0.23±0.24	13.87±3.95	8	0.05±0.05	11.44±1.56	15	1.00±1.47	8.21±2.06
3.5	0.10±0.07	13.07±0.75	10	0.06±0.13	12.73±3.52	21	0.36±0.62	7.82±3.26
4	0.09±0.08	13.09±0.75	13	0.80±1.20	12.57±3.91	27	0.67±1.39	9.75±1.24
6	0.07 ±0.14	12.09±3.41	-			-		

As expected, the fastest growth of *E. coli* O157:H7 was observed at 16 ºC (Figure 3). All samples of lettuce presented growth of *E. coli* O157:H7 after 1 day of storage. Population densities increased up to 7.04. log cfu/g in the first 3 days (i.e. an increase of 3 log CFU over the inoculum at day 0), and after reaching stationary phase, counts slightly decreased to a level of 5-6 log cfu/g. Unlike low storage temperatures, growth variability between repetitions was much lower, situated in <1 log cfu/g between samples, which is quite close to the typical plate count error. This observation hinted that variability was reduced at temperatures more conducive to growth of the bacterium.

Primary model and kinetic parameters

The best-fit growth model is represented by a black solid line in Figures 1-3 for all growth temperatures. By visual observation, the Baranyi model showed an acceptable

fitting to growth data taking into consideration the high variability shown between replicated samples. The Baranyi model particularly showed better fitting in the growth phase (from which Grmax is derived) while at maximum population densities the model performed worse showing in some cases (i.e. 13 and 16 ºC) significant discrepancies between the best-fit line and observations. Predicted kinetic parameters and statistics associated with the regression analysis are presented in Table 2. The adjusted R^2 was used as an index to assess the goodness-of-fit of the growth models where adjusted R^2 = 1 indicates that the regression line perfectly fits the data. Their values were 0.62, 0.74 and 0.80 at 8, 13 and 16 ºC, respectively with lower temperatures showing worse fitting because of higher variability presented by replicates (Table 2). Confidence and prediction bands were computed and plotted together with the best-fit model and experimental data (Figures 1-3). Overall, the prediction bands contained > 82 % out of the observed data which means that the approach taken to computing such bands was acceptable despite the high variability found between repetitions and analysis points.

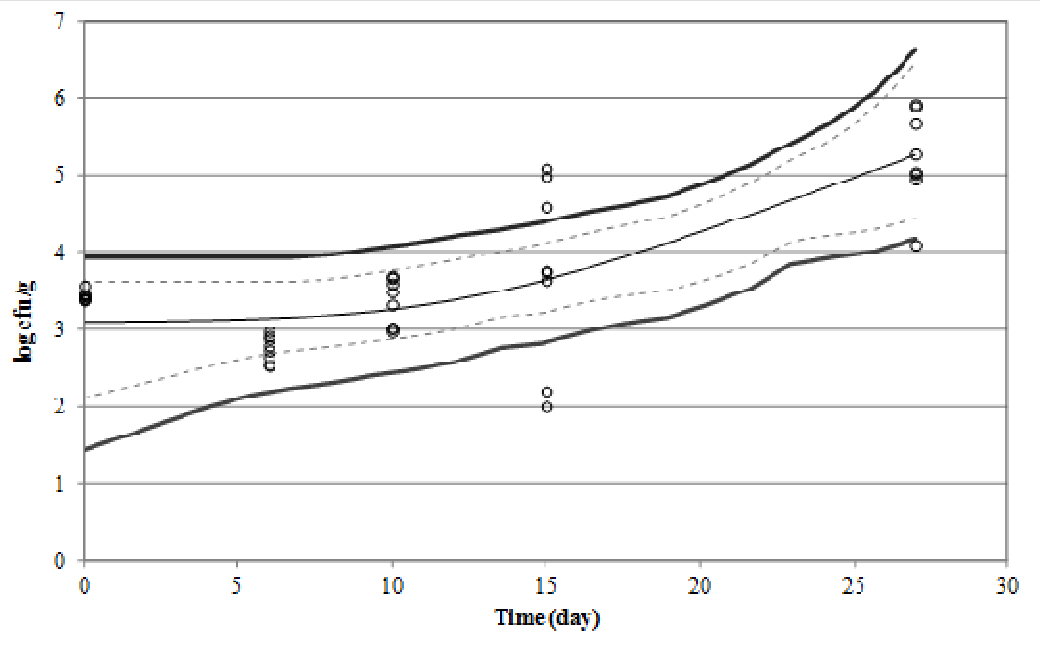

Figure 1. Growth data of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with chlorinated water stored at 8 ºC and prediction curves based on the Baranyi model (Baranyi & Robert, 1994), including best-fit line (inner solid line), and confidence (dotted line) and predictions (outer solid line) bands generated by Monte-Carlo analysis.

At 8 °C, the estimated best-fit value (i.e. mean) for Grmax corresponded to 0.14 log cfu/day and the 95% confidence interval for Grmax was 0.06-0.31 log cfu/day. At this temperature, the lag phase presented a best-fit value of 5.94 days; however, lag phase could be as long as 20 days at the 95th percentile. In Figure 1, confidence and prediction bands are represented by a dashed line and external grey solid line, respectively. The prediction bands indicate the prediction error associated to each time point. These bands comprised a wide range as observed in Figure 1, which was a consequence of the variability in experimental outcomes.

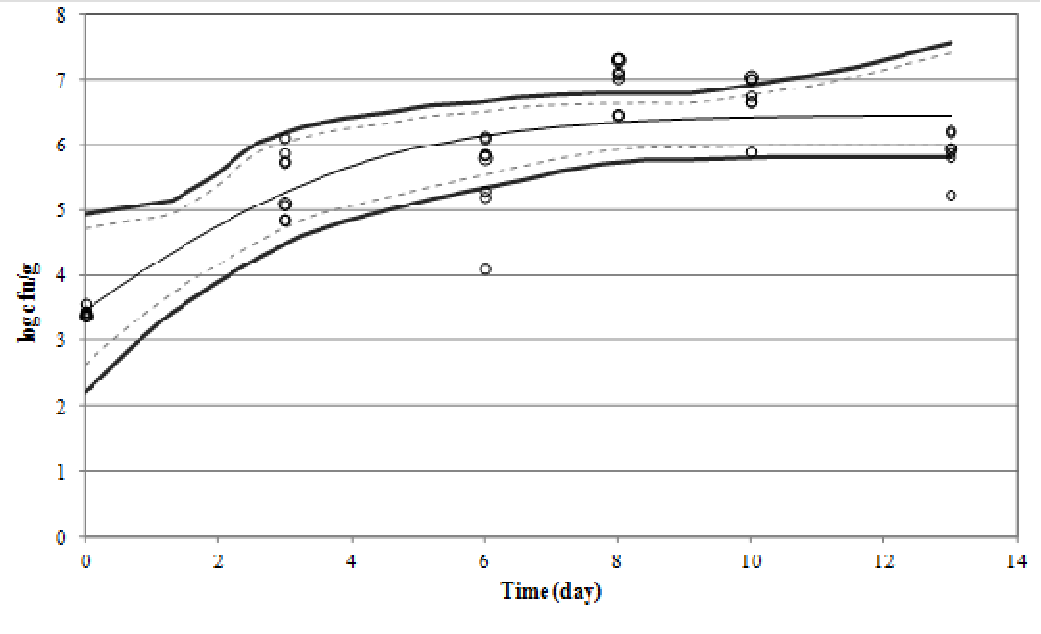

Figure 2. Growth data of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with chlorinated water stored at 13 ºC and prediction curves based on the Baranyi model (Baranyi & Robert, 1994), including best-fit line (inner solid line), and confidence (dotted line) and predictions (outer solid line) bands generated by Monte-Carlo analysis

When samples were stored at 13 ºC, estimated Grmax increased to 0.55 log cfu/day, approximately three times the Grmax obtained at 8 ºC (Table 2). The 95 % confidence interval (CI) for this kinetic parameter was situated in the range 0.17-1.22 log cfu/day (Table 2). Besides that, at 13 ºC, no lag was estimated by the Baranyi growth model. This means that in two and a half days, *E. coli* O157:H7 population might increase 1.5 logs cfu/g. Regarding the prediction bands, these spanned a wide zone, indicating again the great variability in growth data.

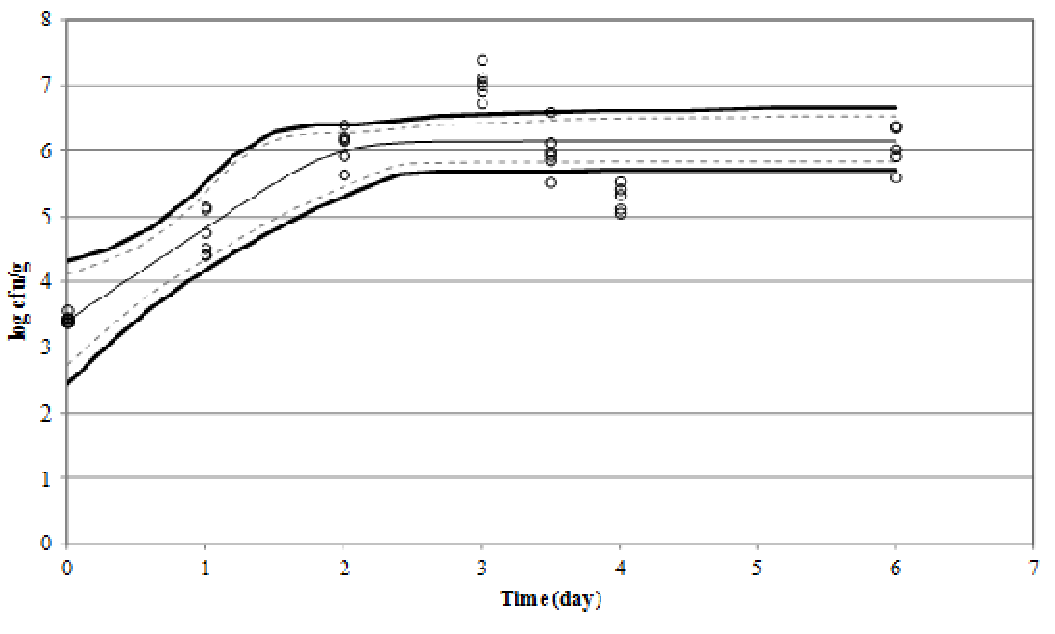

Figure 3. Growth data of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with chlorinated water stored at 16 ºC and prediction curves based on the Baranyi model (Baranyi & Robert, 1994), including best-fit line (inner solid line), and confidence (dotted line) and predictions (outer solid line) bands generated by Monte-Carlo analysis.

At 16 ºC, growth model estimated a Grmax of 1.43 log cfu/day with a 95% CI corresponding to 0.82-2.15 log cfu/day. With respect to prediction bands (see Figure 3), growth data at 16 ºC enclosed a narrower zone than at lower temperatures, probably derived from lower growth variability observed at higher temperature. In spite of this fact, the 95 % CI for Grmax at 16 ºC was larger than those obtained at lower temperatures (8 and 13 ºC) as a consequence of the magnitude of growth which was greater at high temperatures. This means that uncertainty on predictions at 16 ºC results in major differences in growth (Δ [95% CI] = 1.32 log cfu/day) than predictions at 8 ºC

A secondary model for predicting growth rate of *E. coli* O157:H7 as function of storage temperature in leafy green products

The square-root model described by Ratkowsky et al. (1982) was satisfactorily fitted to square root growth rates ($\sqrt{Gr\ max}$) as shown by the coefficient of determination (R^2) and the Standard Error (SE) which were equal to 0.81 and 0.16, respectively. The value estimated for the regression parameter b was 0.104 (95% CI: 0.096-0.112) and for $T_{min,}$ it was 5.125 ºC (95% CI: 4.533-5.760). The fitted square-root model and its 95 % confidence bands are represented in Figure 4 together with the estimated growth rates obtained in our study and those reported by other studies and predicted by two tertiary models: the stand-alone software US Department of Agriculture-Agricultural Research Service's Pathogen Modeling Program (PMP version 7.0) and the on-line software Combase Predictor (www.combase.cc) developed by the Institute of Food Research (Norwich, UK). The best best-fit line and confidence bands for the fitted secondary model were within the 95 % confidence intervals of growth rates estimated by Monte-Carlo analysis thus corroborating the good fitting reported by the statistical indexes.

Table 2. Statistics for estimated Maximum Growth Rate (Grmax) of the Baranyi growth model for observed growth at 8, 13 and 16 ºC and goodness-of-fit indexes associated with the regression analysis (Δ [95% CI] = 0.25 log cfu/day).

Temperature (ºC)	Grmax (log cfu/day)				R^2-adjusted	Standard Error (S_{yx})
	2.5^{th}	97.5^{th}	Median	Mean		
8	0.06	0.31	0.10	0.14	0.62	0.66
13	0.17	1.22	0.51	0.55	0.74	0.62
16	0.82	2.15	1.40	1.43	0.80	0.53

Moreover, growth data from other studies included as growth rates (log cfu/day) in Figure 4 were in agreement with both our growth observations and the fitted secondary model. Besides that, the model was submitted a validation process by using the Bias factor (B_f) and Accuracy factor (A_f) as described by Ross (1996). With this aim, growth rates for the pathogen in leafy green vegetables in the range 8-16 ºC were collected from different studies (Table 3). A previous work by McKellar and Delaquis (2011) that reported digitalized growth data from those studies was used for such purpose. Among the different studies in Table 3, we highlight the study by Abdul-Raouf et al. (1993) which reported E. *coli* growth at 12 ºC both under air and MAP, reaching ~7 log cfu/g in 3 days.

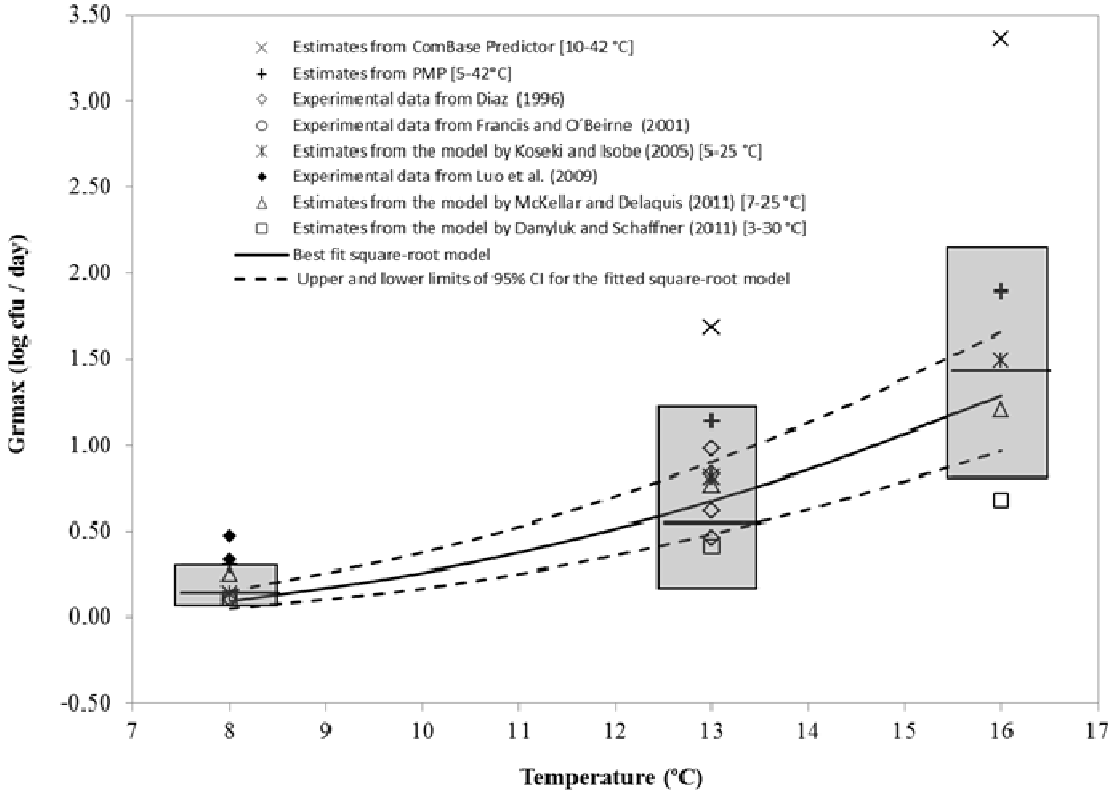

Figure 4. Representation of the fitted square-root model describing Grmax (log cfu/day) as function of temperature and attendant confidence bands. Representation of Grmax values generated in the present work at 8, 13 and 16 º C are included as a box-plot representing 95 % confidence interval and average. Individual points represented by different symbols correspond to Grmax values taken from literature at 8, 13 and 16 ºC.

The growth rate estimated from that study (~0.6 log cfu /day) was within the prediction interval of the Grmax estimated in the present study. Likewise, Li et al. (2001) reported that *E. coli* O157:H7 on shredded lettuce packaged in air and MAP increased by 2.3 to 3.2 log cfu/g within 2 days (i.e. 1.15 and 1.55 log cfu/day) when stored at 15 °C (Table 3). These data are similar to those obtained in our study for 16 ºC, since Grmax was situated in 1.43 log cfu/day (95 % prediction interval = 0.82-2.15 log cfu/day). However, in this case, results from Li et al. (2001) were obtained on lettuce treated with water containing only 20 mg/L chlorine. The obtained B_f and A_f corresponded with 0.97 and 1.46, respectively. These values confirmed that the developed model could predict, in the range 8-16 ºC, *E. coli* O157 growth with accuracy, even though B_f value (< 1) indicated that model slightly underestimated growth on the basis of the used growth studies.

Discussion

E. coli O157 growth in leafy green vegetables

Previous studies using confocal scanning laser microscopy have shown that cells of *E. coli* O157:H7 that had penetrated into the lettuce leaf tissue at the cut edges, were more likely to show cell viability after treatment with chlorine (Takeuchi & Frank, 2000; Takeuchi et al., 2001). Although the recovery of *E. coli* O157:H7 subjected to stress conditions has been documented (Auer, 2009; Kolling & Matthews, 2001), few

studies have considered the assessment of *E. coli* O157:H7 growth after disinfection treatment with chlorine.

Table 3. Growth data sources used to validate the growth model developed for *E. coli* O157:H7 in fresh-cut lettuce.

Source	Food matrix	Temperature (ºC)	Commercial treatment
Addul-Raouf et al. (2003)	Lettuce	12	MAP*
Delaquis et al. (2002)/ McKellar and Delaquis (2011)	Lettuce	10	Heat treatment
Delaquis et al. (2007)/ McKellar and Delaquis (2011)	Lettuce	15	Non-treatment
Diaz and Hotchkiss et al. (1996)	Lettuce	13	MAP
Francis and O´Bernie (1996)	Lettuce	8	Non-treatment
Lie et al. (2001)	Lettuce	15	Chlorine
Luo et al. (2009)	Spinach	8 and 12	Non-treatment
Koseki and Isobe (2005)	Lettuce	10 and 15	Non-treatment

*MAP: Modified Atmosphere Packaging

A study by Kolling & Matthews (2007) showed that starved *E. coli* O157:H7 treated with chlorine grew more rapidly than non-treated *E. coli* O157:H7 presenting shorter lag phase and higher growth rate. Another study targeting aerobic mesophilic microorganisms and applying predictive models (Lu et al. 2006) found that treatment with chlorinated water could effectively reduce the number of total bacteria on fresh-cut lettuce more than 2 logs and resulted in a lower maximum specific growth rate, but shortened the lag time of the bacteria growth on fresh-cut lettuce.

Storage at 4 ºC produced a slight decrease of the *E. coli* O157:H7 population after 15 days of storage. Other studies also observed a slight decrease of E. coli O157:H7 after storage for non-sanitized and sanitized produce, reporting log-decreases of ≤ 1 log cfu/g (Li et al. 2001; Olivera et al. 2010; Delaquis et al. 2002).

According to Figure 4, Grmax values from our study were in concordance with growth data reported by other studies for the pathogen at the same temperatures. Despite that those studies were performed under different experimental conditions (e.g. no chlorine treatment), most data were included within the 95 % CI because of large variation shown by growth data in our study. Results demonstrate that *E. coli* O157:H7 is able to grow at 8 ºC under anaerobic conditions (i.e. < 1 kPa O_2; 8-14 kPa CO_2) after chlorine stress. In this respect, Francis & O'Beirne (2001) also reported that an O_2-depleted atmosphere containing approximately 10–20 kPa CO_2 had no effect on the growth of *E. coli* O157:H7 in packaged lettuce. The Figure 4 shows that Grmax values at 8 ºC taken

from other studies (Luo et al., 2009; Francis & O'Beirne, 2001) are in agreement with our results being quite close to the 95 % CI. Conclusions derived from previous studies (summarized in Delaquis et al, 2007) have indicated that *E. coli* O157:H7 can show different response in leafy green when stored at 8 ºC (Table 2), ranging from slight growth to no change or to measurable losses in viability. In this respect, our study found that although the best-estimation for Grmax at 8 ºC corresponded to 0.14 log cfu/day, prediction intervals for this parameter ranged between 0.06 and 0.31 log cfu/day. This behaviour can be explained by the fact that 8 ºC might be the temperature defining the growth/non growth interface for this pathogen (Valero et al., 2010). However we cannot preclude that the variation found for Grmax at 8 ºC might be also due to the large variation in the atmosphere composition of the bags (Table 1). In other studies, no growth was found in any tested sample such as the study published by Delaquis et al. (2002), which did not show any *E. coli* O157:H7 growth at 10 °C for 14 days in untreated shredded lettuce and shredded lettuce treated with cold water containing 100 mg/L of chlorine both packaged under passive atmosphere. Among other possible causes, the use of a high chlorine concentration in our study (i.e. 150 mg/L) could reduce competitive microbiota, favouring the growth of *E. coli* O157:H7 in lettuce (Francis et al., 1999), but no conclusive explanation may be given as the differences in experimental conditions between studies.

Modelling *E. coli* O157 growth in leafy green vegetables

As observed in Figure 4 and indicated by the B_f and A_f, the fitted square-root model was in concordance with growth data reported by other studies at different experimental and processing conditions (MAP, chlorine, temperature, etc.). These similarities suggest that the effect of different treatments and experimental conditions is minimal on E. coli O157:H7 growth variability observed in leafy green vegetables (Danyluk & Schaffner, 2011; Oliveira et al., 2010; Theofel & Harris, 2009). Furthermore, secondary models developed by other authors such as Koseki & Isobe (2005) and McKellar & Delaquis (2011) also predicted within the 95 % CI of our model. As an exception, predictions for 13 and 16 ºC from the model by Danyluk & Schaffner (2011) were lower than estimates from both our model and above-mentioned models. Regarding tertiary models, estimates from PMP and Combase Predictor provided the highest growth estimates. In the case of Combase Predictor, predictions were out of 95 % CI of the model developed in our study. These results were expected since tertiary models are mostly based on models developed in culture media.

Although the prediction range for our secondary model comprises a narrow range of temperature (8-16 ºC), it matches the refrigeration temperatures enabling growth of *E. coli* O157:H7, which can be often observed during domestic storage of fresh-cut vegetables as reported by Carrasco et al. (2007). This makes the secondary model here presented especially adequate to describe growth during domestic refrigeration thus providing more specific predictions in the mentioned range 8-16 ºC.

Importance of confidence interval and prediction limit in application of growth models

Several authors have suggested that variability and uncertainty should be included in prediction of growth in order to help to determine differences between growth data generated in artificial broth and food (Challenge-test) or to validate predictive models in food (Geysen et al., 2007, Pouillot et al., 2003). Increasing replicates in challenge tests and deriving predictions intervals from those can help to take into consideration the real behaviour in foods, making more reliable models and endorsing more suitable

management decisions. Results in our study showed that growth variability was larger at lower temperature, even though the high temperatures also presented a considerable variability. This variation should be considered if wanting to generate suitable models or compare the effect of different disinfection treatments. This information is also very valuable for incorporation in risk assessment procedures or stochastic models, in which prediction intervals should be considered for the different kinetic parameters (Oscar, 2004; Poschet et al., 2003; Nauta, 2000).

Conclusions

In summary, when simulating commercial processing conditions including chlorine washing and MAP, *E. coli* O157:H7 growth was strongly influenced by temperature as evidenced by the estimated maximum growth rates, which increased as the temperature augmented. Experimental variability might be captured by using a high number of replicates, revealing that *E. coli* O157:H7 can present variable response for similar experimental conditions, especially at low temperatures (8 ºC) in which stressing conditions are more severe. Although in some cases, no growth occurred at 8 ºC, risk is still high due to the highly infectious properties of *E. coli* O157. A specific secondary model describing growth rate as function of temperature was derived from our growth observations, showing a good agreement with literature data and analyzed predictive models. Hence, this model might to be satisfactorily applied to describe *E. coli* O157 growth in domestic refrigeration temperatures (8-16 ºC). The confidence and prediction bands generated for the fitted growth models may be helpful to compare and validate existing predictive models for *E. coli* O157:H7, and subsequently to be efficiently included in quantitative risk assessment studies.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7) under grant agreement no 244994 (project VEG-i-TRADE, www.veg-i-trade.org), the MICINN (projects AGL2010-20070 and AGL2008-03298), the United States Department of Agriculture with the Grant GEO-2007-01899 and the Excellence Project AGR-01879 (Junta de Andalucía). Special thanks to the Research Group AGR-170 HIBRO of the “Plan Andaluz de Investigación, Desarrollo e Innovación” (PAIDI), International Campus of Excellence in the AgriFood Sector ceiA3. M.V. Selma is holder of a Ramón y Cajal contract and G.D. Posada-Izquierdo is holder of a predoctoral scholarship.

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Chapter IV: "Modeling Growth of *Escherichia coli O157*:H7 in fresh-cut lettuce treated with Neutral Electrolyzed Water and under Modified Atmosphere."

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Submitted for published in: International Journal of Food Microbiology, 2013

Abstract

The purpose of this study was to evaluate and model the growth of *Escherichia coli* O157:H7 in fresh-cut lettuce submitted to a neutral electrolyzed water (NEW) treatment, packaged in Modified Atmosphere and subsequently stored at different temperatures (4, 8, 13, 16 ºC) for a maximum of 27 days. Measured counts indicated that *E. coli* O157:H7 was able to grow at 8, 13, and 16 ºC, and declined at 4 ºC. Results were also compared with a previous study dealing with chlorination (Posada-Izquierdo et al., 2013), as disinfection method, developed under the same experimental set-up, indicating that *E. coli* O157:H7 exhibited a lower growth when NEW was used. For example, at 8 ºC, the lag time lasted 19 days in samples treated with NEW, while for the chlorinated water (Posada-Izquierdo et al., 2013), the lag time was lower than 6 days. A secondary model predicting growth rate as a function of temperature was developed based on a square-root function. Comparison with literature data indicated that growth predicted by the model for *E. coli* O157:H7 was again lower than those observed with other disinfection treatments or packaging conditions (chlorinated water, untreated product, NEW, etc.). The specific models here developed might be applied to predict grow in products treated with NEW and to improve existing quantitative risk assessments.

Introduction

Nowadays due to the increasingly stressful lifestyle, people dedicate less time to routine tasks as cooking. At the same time, the population is getting more aware of the importance of an appropriate diet for health and wellbeing. This socio-cultural framework explains for the great demand forready-to-eat (RTE) minimally processed fruits and vegetables, because these food products combine healthiness with easy and fast preparation.

Although the prevalence of bacterial pathogens in fruits and vegetables is low as compared with other food products (Doyle and Erickson, 2008; Francis et al., 1999; Garg et al., 1990; Gómez-López et al., 2008), RTE fruits and vegetables can represent a potential health risk due to the fact that no heat treatment is included in their production chain, and the only step aimed to reduce the microbial load are washing and sanitizing treatments (Artés et al., 2009; Beuchat, 2002). Recent outbreaks linked to the consumption of RTE vegetables include cases of Escherichia coli O157: H7 in Denmark (2010), in the Netherlands (2007) and in Sweden (2005), and the recent and notorious E. coli O104 Outbreak in Germany (2011), and (Wu et al., 2011). Apart from the public health consequences, food-borne outbreaks can also lead to important losses in the food industry, due to changes in consumer confidence, and effects in trade flows consequence of political decisions (De Vocht et al., 2011).

In the production of RTE vegetables, sodium hypochlorite is the most widely used sanitizer (Akbas and Ölmez, 2007; Behrsing et al., 2000). However, concerns related to its efficacy and the formation of potentially hazardous by-products have provoked an intense research effort aimed to find alternative treatments more effective and that could generate lower quantities or less harmful disinfection by-products. One of the proposed alternatives is to use electrolyzed water (EW) as sanitizer (Guentzel et al., 2008). EW is obtained by conducting an electric current through water containing dissolved NaCl. During this process, electrolysis takes place producing oxidizing agents with strong antibacterial activity such as hypochlorous acid (Artés et al., 2009). This technique gets similar results using lower concentrations of free chlorine, and as a consequence, less disinfection by-products would be generated (Alegria et al., 2009; Al-Haq et al., 2005; Chang et al., 2000). There are different types of EW depending on if solution is formed in the anode, which is called acidic electrolyzed water (AEW), acid oxidizing water, or electrolyzed oxidizing water or in the cathode which is known as basic electrolyzed water (BEW), alkaline electrolyzed water, or electrolyzed reducing water. Neutral electrolyzed water (NEW), with a pH around 7, is produced by mixing the anodic solution with an alkaline solution or by using an only single-cell chamber, without separation between both electrodes (Hricova et al., 2008). In spite of the need of predicting growth of *E. coli* O157:H7, with accuracy, in leafy green vegetables, to date few predictive models have been developed on the own food matrix (i.e. vegetables). Koseki and Isobe (2005) developed a model for *E. coli* O157:H7 growth on non-packaged iceberg lettuce as a function of temperature (5-25 °C) and more recently, a growth model for the pathogen in fresh-cut lettuce submitted to washing with chlorinated water and packaged in modified atmosphere (Posada-Izquierdo et al., 2013). In addition, two growth models based on scientific literature data on growth of *E. coli* O157:H7 in fresh-cut leafy vegetables have been developed (Danyluk and Schaffner, 2011; McKellar and Delaquis, 2011). These models consider growth data from different leafy green vegetables and under different processing conditions thus producing a more general model. Anyhow, it would be expected that different processing conditions lead to different growth patterns during storage. Therefore, specific models assessing the growth of pathogens after different decontamination treatments would be needed in order to perform more accurate quantitative risk assessments on RTE vegetables. The aim of this study was to evaluate and model the effect of a treatment with neutral electrolyzed water on the subsequent growth/survival of *E. coli* O157:H7 in modified atmosphere packaged lettuce at different storage temperatures.

Materials and methods

Bacteria and preparation of cell suspensions

In the present study, a five-strain cocktail of *Escherichia coli* O157:H7 (CECT 4267, 4076, 4782, 4783, and 5947) provided by the Spanish Type Culture Collection (CECT, Valencia, Spain) was used. Previously, each strain was made resistant to 50 μg of Nalidixic acid (Nal^R strains) per mL (Merck, Darmstadt, Germany), by transferring and incubating (37º C; 20 h) the microorganism successively in Brain Heart Infusion (BHI, Oxoid, Basingtoke, UK) tubes with increasing concentrations of the antibiotic.

To prepare the five-strain cocktail for experiments, Nal^R strains were separately subcultured twice at 37 °C for 20 h in 5 mL of BHI with Nalidixic acid (Nal). In order

to remove presence of BHI, cultures were centrifuged at 4100 rpm for 10 min, and the supernatant was removed and replaced by0.1% $NaHCO_3$ solution. This procedure was repeated three times. Afterward, suspensions of each strain were mixed in equal volumes to have the same concentration of each of the strains (approximately, 10^9CFU/mL). Levels of *E. coli* O157:H7 in the inoculum were confirmed by using Chromocult coliform agar (Merck, Barcelona, Spain) supplemented with 50 µg Nal per mL agar (Nal^+) incubated at 37 °C for 24 h.

Inoculation of Fresh produce

Iceberg lettuce (*Lactuca sativa L.*) acquired from a local market in Murcia, (Spain) was processed under commercial conditions at 4 °C. Outer and damaged leaves were manually removed, and the rest was cut in pieces of 3x3 cm, approximately. Afterwards, it was inoculated by immersion for 1 min in 10 L of cold (4 °C) tap water with a concentration of $5 \cdot 10^6$CFU/mL of the cocktail of Nal^R *E. coli* O157:H7.Subsequently, lettuce was centrifuged to eliminate the excess of water by means a manually-operated enclosed spinner centrifuge (Paragourmet 90005), and kept at 4 °C for 1 hour before the disinfection treatment in order to facilitate attachment of the pathogen on the lettuce tissue. As a consequence of this process, an inoculum of 5 log CFU/g was obtained on lettuce just before the disinfection treatment.

Decontamination treatments

Neutral Electrolyzed water (NEW) was generated by a pilot scale prototype provided by the company Adamant Technologies (La Chaux-de-Fonds, Switzerland). A constant flow of cold water (4 ºC) with a concentration of 1 g/L NaCl (Merck, Barcelona, Spain) was pumped through an electrolytic cell with Boron-doped diamond (BDD) coated electrodes. Amperage was kept at a level of 6.4 A, and the current density applied was 24 mA/cm^2. The obtained solution was diluted with tap water in order to prepare a volume of 40 L of a solution with a concentration of 50 mg/L free chlorine, a pH of 6.5 (adjusted with citric acid to improve disinfection efficacy of chlorine) Oxidation reduction potential (ORP) of >450 mV (López-Gálvez et al., 2012) and a temperature of 4 °C. Aliquots of 3.5 kg of inoculated lettuce were washed for 30 seconds in 40 L of the EW solution, and rinsed with 40 L of cold tap water (4 ºC) also for 30 s. Finally, the excess of water was removed with the same manual centrifuge mentioned above, by applying 50 rpm for 1 min, approximately.

Temperature, pH, and ORP in the wash-water were measured by means of a multimeter pH & Redox 26 (Crison, Barcelona, Spain), whereas free and total chlorine were determined based on the N,N-diethyl-p-phenylendiamine (DPD) method (APHA, 1998) using the Spectroquant NOVA 60 photometer (Merck, Darmstadt, Germany).

Packaging and storage

Samples of 50 g of treated lettuce were packed in passive modified atmosphere in 12x12 cm bags simulating commercial proportions for 250 g bags of 23x30 cm. Bags were made of oriented polypropylene (OPP) with a thickness of 35 µm and a permeability to O_2 of 1100 $mL/m^2 \cdot day \cdot atm$ at 23 ºC. An amount of 56 bags were stored at each temperature (8, 13 and 16 ºC) in controlled temperature chambers (Tecnidex, Valencia, Spain). Additionally, a smaller amount of bags was stored at 4 °C. During the storage, temperature in the chambers was controlled every 30 min. The evolution of gas composition inside the bags was measured with a gas analyzer (Systech Instruments GASPACE 2, 5979, London, UK) each sampling time before microbiological analysis.

Microbiological analysis

Sampling was performed on time points, which were determined based on preliminary experiments (data not shown). Sampling points were more distant in time at low temperatures, and were closer at high temperatures. At each sampling point, 8 bags were taken from each temperature to be analyzed for *E. coli* 057:H7. Each sample consisted of the whole content of one bag. Therefore, 50 g of lettuce were homogenized with 200 mL of buffered peptone water (Scharlau, Barcelona, Spain) for 2 min in a stomacher (IUL Instruments, Barcelona, Spain), then plated in Chromocult coliform agar supplemented with Nal using a spiral plater (model WASP, DW scientific, Shipley, UK). Plates were incubated for 24 hours at 37 °C before counting in an automated plate counter (ProtoCOL, Synoptics, Cambridge, UK).

Mathematical analysis

Microbial concentration levels were log-transformed and introduced in Excel spreadsheet (raw data). The primary growth model of Baranyi and Roberts (1994) was fitted to the raw growth data by means of DMFit excel Add-In (Institute of Food Research, Norwich, UK), which estimates three kinetic parameters, i.e. lag time (k, expressed in days in this paper), maximum growth rate (Gmax, expressed in log CFU/day) and maximum cell numbers (yend). The fitting procedure used was specific for repeated measures, which were the concentration values (log) obtained in eight replicates analyzed at each time point.

The Confidence interval (CI) for each estimated kinetic parameter (Grmax, yend and lag) was computed based on Monte-Carlo method using the Standard Error (S_{xy}) reported by the DMFit program. The CI provides the uncertainty range on the estimated regression parameters at a certain level of confidence or probability. To compute the CIs for the kinetic parameters, a normal distribution was defined with mean= 0 and SD= S_{xy} accounting for the range of error in the estimated concentration (log CFU/g) associated with the best-fit estimate for the Baranyi model. Then, normal distributions defined at each time point were simulated by using Monte-Carlo methods with 1000 iterations for each one, resulting in a set of new 1000 growth curves. With the DMFit program, the Baranyi model was fitted to each new growth curve obtained by Monte-Carlo simulation obtaining different estimates of the kinetic parameters for each growth curve. The 95 % CI for each kinetic parameter was obtained based on 2.5^{th} and 97.5^{th} percentile values for each set of simulated kinetic parameter values. In addition, the obtained growth 2.5^{th} and 97.5^{th} percentiles at each time were plotted to represent the upper and lower limits of 95 % CI for the estimated best-fit growth model.

Secondary model and validation

The secondary model corresponding to the root-square model by described Ratkowsky et al. (1982) was chosen to describe the effect of temperature on maximum growth rate (Grmax). The used model function was as follows:

$$\sqrt{Gr\max} = \mathrm{b} \cdot (T - \mathrm{T}_{\min})$$

where b and T_{min} are regression parameters. The latter is considered the conceptual minimum temperature for microbial growth.

The model was fitted to Grmax values from Monte-Carlo method with least square linear regression using the software Statistica 10.1 (StatsoftIberica, Lisbon, Portugal) and 95 % CI were computed and plotted together with the best-fit growth model. The

goodness of fitting was evaluated by applying the coefficient of determination (R^2) and Standard Error (SE). For validation purposes, model predictions were compared with growth data from literature by using Bias factor (B_f) and Accuracy factor (A_f) described by Ross (1996).

Results and Discussion

The electrolyzed water treatment was effective in the reduction of the initial level of *E. coli* O157:H7, achieving a reduction of ~1.01 log CFU/g. Therefore, after disinfection, the level of inoculated bacteria in the product surface was 3.65 ± 0.10 log CFU/g). Presence of high levels of surviving cells after the disinfection treatment (i.e. ~3 log CFU/g) was expected since it is assumed that specific areas such as cut edges provide bacterial populations protection from the disinfectants (Annous et al., 2006, 2009). Furthermore these areas permit the growth of microorganisms during storage of the product (Brandl, 2008).

In a previous study performed by our research group (Posada-Izquierdo et al., 2013), disinfection by chlorinated water with 150 mg/L free chlorine under the same experimental set-up produced a similar reduction of initial loads of the pathogen, i.e. 1.21 log CFU/g. These results are consistent with those shown by other authors that suggest that E. coli reduction by electrolyzed water is similar or higher as compared to chlorination treatments (i.e. NaClO-based treatments) performed with lower concentrations of free chlorine (Issa-Zacharia et al., 2011; Jeong et al., 2009; Thorn et al., 2012). Most studies have been conducted to evaluate the bactericidal activity of Acidic EW (AEW) on a variety of microorganisms (Hao et al., 2012; Kim et al., 2000, 2001;Kiura et al., 2002; Park et al., 2001). Abadias et al. (2008); Lindsey et al. (2009) and Park et al. (2001); obtained less than 1 log reduction with approximately the same dose of free chlorine in AEW.

Table 1.Gases composition measured in fresh-cut lettuce bags inoculated with *Escherichia coli* O157:H7 and treated with neutral electrolyzed water over storage at different temperatures (8, 13 and 16 ºC).

8 °C			13 °C			16 °C		
Days	O_2 (%)	CO_2 (%)	Days	O_2 (%)	CO_2 (%)	Days	O_2 (%)	CO_2 (%)
0	22±0.00	0.33 ± 0.0	0	22±0.00	0.33±0.00	0	22±0.10	0.33±0.10
6	0.54±0.38	9.20±1.25	3	1.19±2.14	12.04±1.03	1	3.72±1.63	10.18±1.35
10	1.7±1.94	9.15±0.78	6	0.82±1.98	12.66±2.02	2	3.16±2.45	11.64±1.36
15	0.46±0.41	9.03±1.53	8	0.40±0.76	12.37±1.86	3	0.09±0.03	11.38±0.42
21	0.04±0.07	7.48±2.37	10	0.19±0.28	13.89±4.16	3.5	0.52±0.54	12.03±0.84
27	0.06±0.08	9.38±0.97	13	0.02±0.06	12.74±3.07	4	0.54±0.75	13.34±1.45
						6	0.08±0.09	10.71±1.77
						10	0.00±0.01	14.63±1.90

Gaseous atmosphere inside the packages, which was initially equivalent to the typical atmospheric composition, reached anaerobic conditions after 3 days of storage for 16 ºC. In this case, the concentration of oxygen and carbon dioxide changed from 22±0.1 % to 0.09±0.03 %, and from 0.33±0.1 % to 11.38±0.42 %, respectively (Table 1). However, for lower temperatures, anaerobic conditions were given after 6 and 15 days for 13 and 8 ºC, respectively (Table 1). Growth data indicate that *E. coli* O157:H7 was able to grow at 8 , 13, and 16 °C after the NEW treatment. On the contrary, at 4 °C, *E. coli* O157 population dropped, decreasing ~1.12 log CFU/g in the first 15 days and

other ~1.05 log CFU/g in the last 12 days of storage (Figure 1).

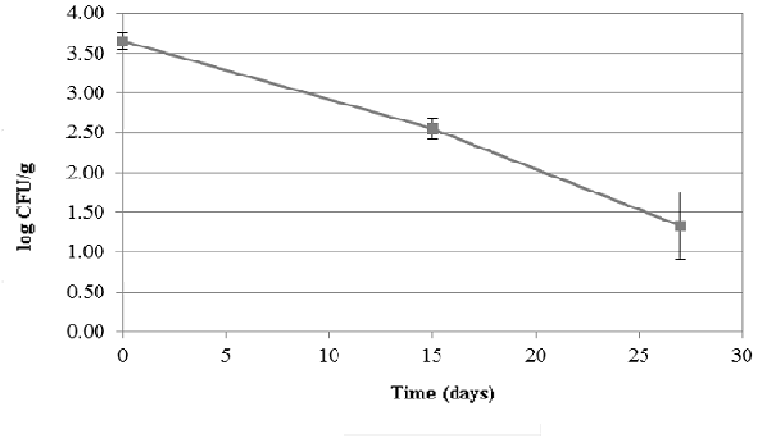

Figure 1. Growth data representing decrease of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with neutral electrolyzed water stored at 4 ºC.

The best-fit growth model is represented by a black solid line in Figures 2-4 for all growth temperatures. In the spite of the high variability shown by samples, the Baranyi growth model showed an acceptable fitting to growth data, specially at high temperatures. Predicted kinetic parameters and regression statistics are presented in Table 2. The R^2-adjusted was used as index to assess the goodness-of-fit of the growth models where R^2-adjusted=1 indicates that the regression line perfectly fits the data. Their values oscillated between 0.40 and 0.78, with the lowest temperature showing (8 ºC) the worse fitting because of higher variability presented by samples. Confidence bands were computed and represented together with best-fit model and experimental data (see Figure 2-4). These bands describe uncertainty around the best-fit growth model as explained in the materials and methods section.

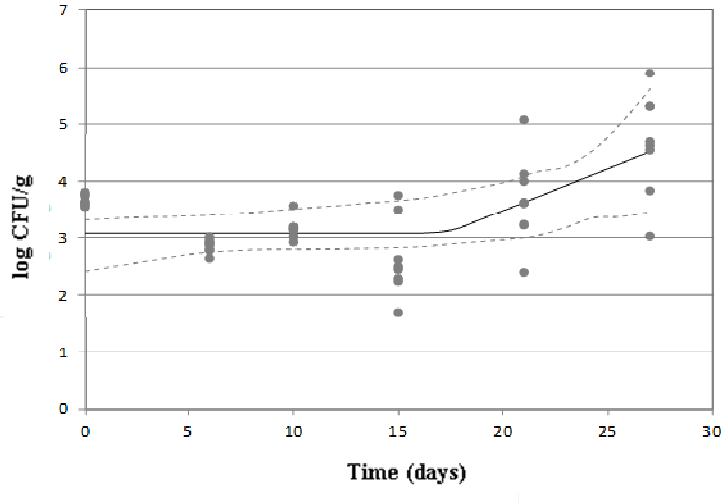

Figure 2. Growth data of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with neutral electrolyzed water stored at 8 ºC and prediction curves based on the Baranyi model (Baranyi and Robert, 1994), including best-fit line and confidence bands.

In samples stored at 8 °C, *E. coli* showed a long lag-phase lasting 17 days, approximately followed by an exponential growth, reaching a mean maximum level of 4.5 log CFU/g although one repetition even contained 6 log CFU/g. These values do not correspond to the population maximum density due to the end of the experiment,

established to 27 days, did not allow observing the stationary phase. The worst fitting obtained for this temperature was due to the high variability among repetitions which was specially evident during exponential growth (15-27 days) with some repetitions remained at the initial or slightly lower levels. The estimated best-fit value (i.e. mean) for Gmax corresponded to 0.25 log CFU/day with CI ranging between 0.04 and 0.79 signaling the high uncertainty on this parameter.

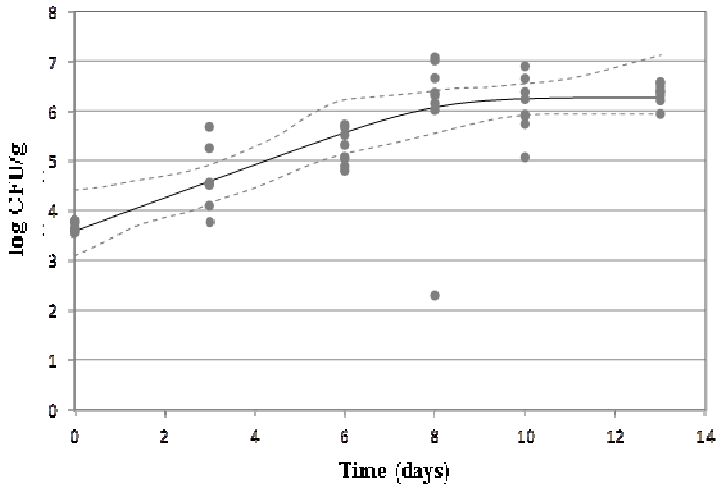

Figure 3. Growth data of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with neutral electrolyzed water stored at 13 ºC and prediction curves based on the Baranyi model (Baranyi and Robert, 1994), including best-fit line and confidence bands.

For samples at 13 °C, no lag was observed, and variability was much lower than at 8 ºC resulting in a better fit to data with the exception of one sample showing lower values than the rest of data points as shown in Figure 3. The best estimate for Gmax was 0.36 log CFU/day with CI= 0.19-1.09 log CFU/day. These values were slightly higher than those obtained at 8ºC. The maximum population density corresponded to 6.24 log CFU/g (CI: 5.93-6.57 log CFU/g). As expected, the fastest growth was observed at 16 °C. At this temperature all samples presented growth at every analysis point. The microorganism was able to grow up to more than 5 log CFU/g in the first 2 days. The estimated Gmax corresponded to 0.94 log CFU/day, nearly three times higher than that obtained at 13 ºC, while the 97.5th percentile for this kinetic parameter was 2.45 log CFU/day (see Table 2).

Table 2. Statistics for estimated Maximum Growth Rate (Gmax) of the Baranyi growth model for observed *Escherichia coli* O157:H7 growth at 8, 13 and 16 °C and goodness-of-fit indexes associated with the regression analysis.
*values between brackets correspond to lag phase (days)

Temperature (ºC)	Gmax (log CFU/day)				R^2-adjusted	Standard Error (S_{xy})
	2.5^{th}	97.5^{th}	Median	Mean		
8	0.04 (12.31)*	0.79 (23.99)	0.22 (19.39)	0.25(19.43)	0.40	0.62
13	0.19	1.09	0.33	0.36	0.78	0.54
16	0.03	2.45	0.79	0.94	0.62	0.55

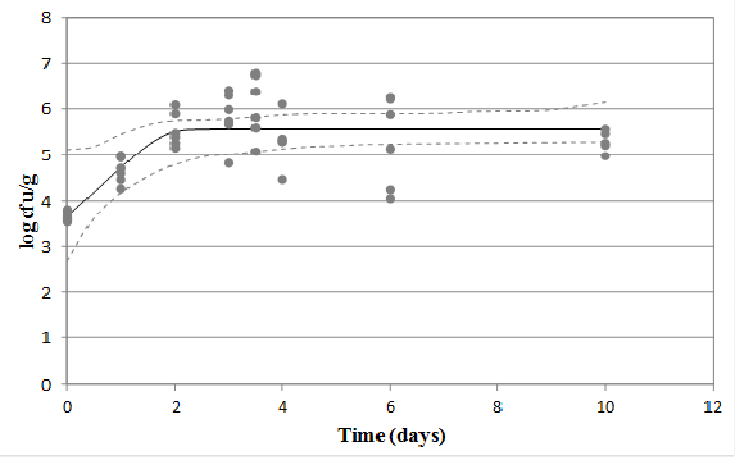

Figure 4. Growth data of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with neutral electrolyzed water stored at 16 ºC and prediction curves based on the Baranyi model (Baranyi and Robert, 1994), including best-fit line and confidence bands.

A secondary model for predicting growth rate of E. coli O157:H7 as function of storage temperature in leafy green products

The fitted square-root model to square root growth rates ($\sqrt{Grmax}$) showed a coefficient of determination (R^2) and the Standard Error (SE) of 0.81 and 0.16, respectively. The value estimated for the regression parameter b was 0.104 (95% CI: 0.096-0.112) and for $T_{min,}$ it was 5.125 ºC (95% CI: 4.533-5.760). In order to assess the capacity of prediction of the secondary model, growth parameters taken from other similar studies (Table 3) were compared to predictions by the best-fit model using as criteria the accuracy and bias factors whose values corresponded to B_f = 0.72 and A_f = 1.67, respectively. The value obtained for B_f suggests that the model predicts lower growth than those in the validation set. Most of the *E. coli* growth data used for validation was obtained from studies with vegetables treated with chlorine or without disinfection treatment (Table 3). This fact might explain for the differences evidenced by B_f, indicating that growth after NEW treatment was lower than that obtained when either chlorine-based disinfection treatment or no disinfection treatment was applied to leafy green vegetables. Only one study, carried out by Smigic et al. (2009), included growth data for vegetables submitted to NEW treatment, considering different storage atmosphere conditions. However, growth rates derived from the study by Smigic et al. (2009) were also higher, and only at 12.5 ºC for atmospheres 60% CO_2/40%N_2 and 30% CO_2/40% N_2, observations were similar to our model predictions (i.e. 0.40 log CFU/day). Although both EW treatments showed some similarities (e.g. neutralized EW and free chlorine), the study Smigic et al. (2009) developed the growth experiments in culture broth hence growth was higher than that observed in our work.

The value for A_f, close to 1, indicated that, though the model underestimated, predictions were quite close to values of the validation data set. When the upper limit for square-root model parameters was used instead, validation indexes were much higher, with B_f = 2.58 and A_f = 3.59. This means that model predictions at the upper limit over-predicts growth, and greatly reduced accuracy of growth predictions. Besides that, growth rates obtained in our study were compared to prediction given by two tertiary models: the stand-alone software US Department of Agriculture-Agricultural Research Service's Pathogen Modeling Program (PMP version 7.0) and the on-line software Combase Predictor (www.combase.cc) developed by the Institute of Food

Research (Norwich, UK). Results indicated that Combase predictor predicted growth rates three and four times higher than predictions from our model, while PMP reported growth values two times higher.

Table 3. Growth data sources used to validate the growth model developed for *Escherichia coli* O157: H7 in fresh-cut lettuce treated with neutral electrolyzed water.

Source	Food matrix	Temperature (ºC)	Commercial treatment
Abdul-Raouf et al., (2003)	Lettuce	12	MAP*
Delaquis et al., (2002)/ McKellar and Delaquis, (2011)	Lettuce	10	Heat treatment
Delaquis et al., (2007)/ McKellar and Delaquis, (2011)	Lettuce	15	Non-treatment
Diaz and Hotchkiss, (1996)	Lettuce	13	MAP
Francis and O´Beirne, (1996)	Lettuce	8	Non-treatment
Lie et al., (2001)	Lettuce	15	Chlorine
Luo et al., (2009)	Spinach	8 and 12	Non-treatment
Koseki and Isobe, (2005)	Lettuce	10 and 15	Non-treatment
Posada-Izquiedo et al., (2013)	Lettuce	8, 13, 16	Chlorine & MAP
Smigic et al., (2009)	Broth	10, 12.5, 15	NEW & MAP

MAP: Modified Atmosphere Packaging
**NEW: Neutralized Electrolyzed Water

Comparison of growth of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with chlorinated water and neutral electrolyzed water at 8, 13 and 16 ºC

Data from another study previously performed for iceberg lettuce treated with chlorine under the same conditions (Posada-Izquierdo et al., 2013) have been compared with the results of the present study (Figure 5). By considering growth data obtained from the same experimental conditions (product, inoculation process, facilities, incubation conditions, equipment, etc) enables a better comparison between both types of treatment, providing more reliable conclusions in relation to the effect of the disinfection treatment on the subsequent growth of the pathogen. Figure 5 A, B, C showing growth curves obtained at different temperatures for both treatments (i.e. chlorine and NEW) indicates that *E. coli* O157:H7 on cut-fresh lettuce submitted to NEW treatment (50 mg/L free chlorine) grew slower than when it was submitted to a chlorination treatment (150 mg/L free chlorine). Although at 8ºC, the microorganism in samples with NEW treatment exhibited a higher growth rate (i.e. 0.25 log CFU/day), the lag time was much longer (19.43 days) than that derived from samples treated with chlorine (5.94 days) (Figure 5 A), which means that *E. coli* O157:H7 in samples submitted to NEW treatment started much later than in the case of chlorine. This fact resulted in lower population densities during the last 20 days of the experiment in

samples treated with NEW. In 13 and 16 ºC, samples treated with NEW experienced lower growth rates and maximum population densities than those observed for samples treated with chlorine as shown in Figure 5 B and C, respectively. Thus, the best fit maximum growth rates obtained for samples submitted to NEW treatment were 0.36 and 0.94 log CFU/day, while for chlorine, the values were 0.55 and 1.43 log CFU/day, respectively. In the case of the maximum population density, the difference was more evident for 16 ºC, with values of 5.60 and 6.17 log CFU/g for samples treated with NEW and chlorine, respectively.

The different growth patterns might be derived from the type of disinfection method applied since the rest of experimental conditions were the same for both experiments. In this sense, the NEW generator used in our work was based on Boron-doped diamond (BDD) electrodes. Studies indicate that BDD generates important amounts of reactive oxygen species (ROS) together chlorine compounds (Cl_2, HOCl, and OCl^-) (Gusmão et al., 2010; Martínez-Huitle and Brillas, 2008). The large amounts of ROS such as production of hydroxyl radicals during water electrolysis result in faster bacterial abatement (Gómez-López et al., 2013; Li et al., 2010; Martínez-Huitle and Brillas, 2008). In this respect, some researchers have also pointed out that the disinfecting efficacy of this method is much higher than that of chlorination method because other oxidants are also formed by electrogeneration (López-Gálvez et al., 2012; Martínez-Huitle and Brillas, 2008; Venczel et al., 2004). Therefore, based on these data, it is likely that cell damage (or/and number of injured cells) is higher in NEW than in chlorination methods due to the higher content of ROS and other unknown oxidants also generated during EW formation process (Feliciano et al., 2011). In this sense, lag time is considered a measure of the time needed to repair sublethal injury hence the longer lag time observed at 8 ºC in NEW samples could derive from a higher number of sublethal injured cells (Dukan and Nystrom, 1998; Mackey and Derrick, 1982). Also, growth rates were lower at higher temperatures in NEW, which could be due to a residual effect of remaining oxidizing species from the NEW after dewatering. However, more specific experiments looking at the type of generated oxidizing agents and quantifying the number of injured cells (by using microscopy analysis or more advanced molecular methods) should be performed to draw more definitive conclusions on this hypothesis.

The secondary models generated for both conditions are plotted in Figure 5 D, here can be observed that both differed in its best fit line, even though the secondary model for NEW showed wider confidence bands, which could be consequence of the higher variability found among samples. Although most part of the predictions derived from the model for chlorine are included in the wide confidence interval of the model for NEW, both models should be considered different as they accounts for both different physiological states of cells (i.e. cell injury) and growth behavior, with a better growth capacity of *E. coli* O157:H7 when chlorine treatment (150 mg/L free chlorine) is applied. Therefore, predictive microbiology practitioners should not only choose those models that better fits the phenomenon to be represented but also those considering previous conditions or technological treatments (i.e. chlorinated water or NEW) affecting physiological state of cells, and therefore the subsequent behavior of microorganisms (i.e. pre-culture conditions).

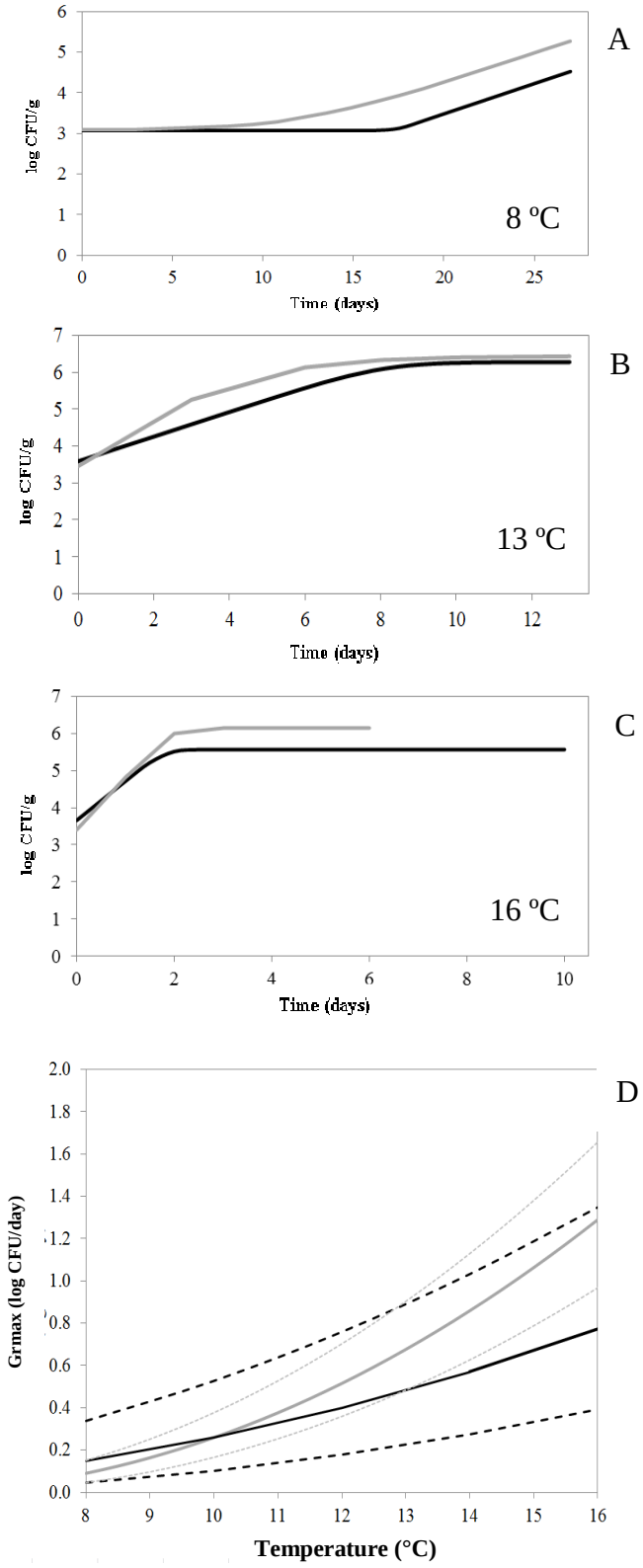

Figure 5. Graphical comparison of primary (A, B, C) and secondary (D) growth models of *Escherichia coli* O157:H7 in packaged fresh-cut lettuce treated with chlorinated water (grey line) reported by Posada-Izquierdo et al. (2013) and with neutral electrolyzed water (black line) developed in the present work.

Conclusions

In summary, data suggest that *E. coli* O157: H7 is able to survive on lettuce after treatment with neutral electrolyzed water (NEW), and then to grow at storage temperatures ≥ 8 ºC. When comparing with the growth after chlorinated water treatment, *E. coli* O157:H7 grew slower at all studied growth temperatures after treatment with electrolyzed water. The lag time was longer at 8 ˚C, it finished after around 19 days, above the typical expiry date given to fresh-cut leafy greens (10-15 days). Therefore products treated with NEW would not show pathogen growth, if kept at 8 ºC or below. On the contrary, non-extreme abuse temperatures like these studied in this work (13 and 16 ºC) would allow an immediate and larger growth of the pathogen. This finding highlights the importance of maintaining an adequate temperature control during distribution and storage since disinfection treatments are not completely effective in eliminating pathogens in vegetables. Furthermore, the predictive model derived herein provides suitable growth predictions for *E. coli* O157:H7 in leafy green vegetables submitted to a NEW treatment and packaged under MAP. The lack of predictive models for such specific conditions makes this model particularly relevant to be applied in risk management or to be included in quantitative risk assessment studies.

Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7) under grant agreement no 244994 (project VEG-i-TRADE, www.veg-i-trade.org), the MICINN (projects AGL2010-20070 and AGL2008-03298)co-funded by the European Regional Development Fund (ERDF), the United States Department of Agriculture with the Grant GEO-2007-01899 and the Excellence Project AGR-01879 (Junta de Andalucía). Special thanks to the Research Group AGR-170 HIBRO of the "Plan Andaluz de Investigación, Desarrollo e Innovación" (PAIDI), International Campus of Excellence in the AgriFood Sector ceiA3. G.D. Posada-Izquierdo is holder of a predoctoral scholarship.

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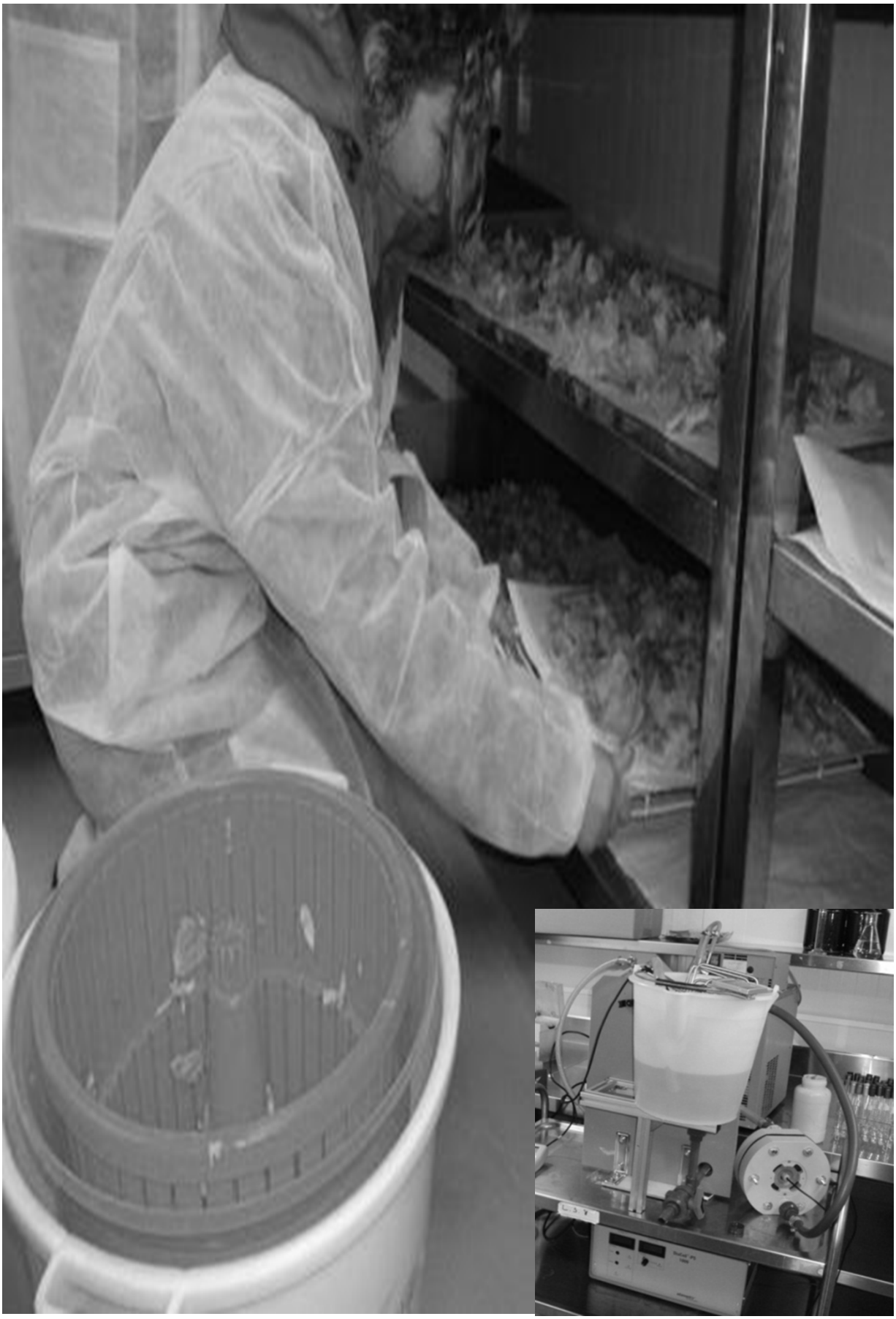

Chapter V: "Modelling Survival of *Escherichia coli* O157:H7 and Salmonella spp. on stainless steel surfaces soiled with different vegetables juice substrates"

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Submitted for publication in: Food Research International, 2013.

Abstract

Microbial survival on inert surfaces should be considered as an important factor to understand and quantify bacterial transfer to foods (i.e. cross contamination). The present work studied the survival of *Escherichia coli* O157:H7 and *Salmonella* spp. on stainless steel surfaces with different soiling substrates based on leafy vegetable juices. Furthermore, predictive models were proposed to describe survival patterns shown by both pathogens in the different substrates. *E. coli* O157:H7 and *Salmonella* spp. could be recovered until 192 and 168 h, respectively from surfaces soiled with chard, red cabbage, iceberg lettuce and romaine lettuce. However, in spinach and parsley juice substrates, microorganisms were not detected after 48 h. This survival time was much lower than that obtained in saline solution (120 h) used to simulate cleaning conditions. This result might suggest the presence of antimicrobial substances in both vegetable juices. The log-reduction observed at 2 and 24 h for *E. coli* O157:H7 depended on type of substrate and ranged 2-4.5 log cfu/cm^2 and 3.2-6.5 log cfu/cm^2, respectively. For *Salmonella* spp. results showed slightly lower drops, with 1.7-3.3 and 3.5-6 log cfu/cm^2, for 2 and 24 h, respectively. The Weibull model and Weibull+tail model seemed to be the most suitable mathematical functions describing survival of both microorganisms in the different substrates (adj-R^2=0.94-0.99). Experiments were developed simulating environmental conditions given in Fresh-cut Vegetable Factories. From that, results and models here generated could be applied in Quantitative Risk Assessment studies to obtain a better understanding and estimation of cross contamination, in addition to enabling the assessment of control strategies of both pathogens in the fresh-cut vegetable industry.

Introduction

Cross contamination is a serious concern for food industries as food-borne pathogens can be transmitted from environment and workers to foods during manufacturing and handling (den Aantrekker, Boom, Zwietering & van Schothorst, 2003; Pérez-Rodríguez, Valero, Carrasco, Garcia-Gimeno & Zurera, 2008; Reij & den Aantrekker, 2004). Cross-contamination events are strongly related to the ability of microorganism to survive on the contact surfaces in the food industry (Legnani, Leoni, Berveglieri, Mirolo & Alvaro, 2004; Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, 2013; Todd, Greig, Bartleson & Michaels, 2009; Zhao, Zhao, Doyle, Rubino & Meng, 1998). It has been shown that bacteria are able to remain active for hours or even days, when they are contaminated through contaminated water, soil, insects, hands, clothing, countertops, cutting, etc. (Critzer & Doyle, 2010; Gormley, Little, Grant, de Pinna, & McLauchlin, 2010; Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, 2013; Scott & Bloomfield 1990).

Enteric pathogens such as *Escherichia coli* O157: H7 and S*almonella* spp. are a prime

concern for the ready to eat (RTE) vegetable industry. These microorganisms can be present in raw vegetables (Quiroz-Santiago et al., 2009) and then survive industrial disinfection processes thus reaching consumers because no subsequent lethal treatment is applied (Fuster-Valls, Hernández-Herrero, Marín-de-Mateo & Rodríguez-Jerez, 2008; Aparecida de Oliveira, Abeid, Morato & Pereira De Martinis, 2010). Other relevant contamination cause is cross contamination during industrial process (Lynch, Tauxe & Hedberg, 2009; Olaimat & Holley, 2012). In this sense, not only the washing step is crucial in post-harvest contamination (López-Gálvez, Allende, Selma & Gil, 2009) but also, as evidenced by Buchholz, Davidson, Marks, Todd & Ryser. (2012ª,b) for *Escherichia coli* O157:H7, enteric pathogens could spread, during processing, within the processing facilities, equipment (conveyor belt, centrifuge, flume, etc.) and washing water thereby contaminating production. In fact, according to (FAO/WHO, 2008) vegetable industries should be divided into dirty and clean zone area (or white) in order to minimize the possible causes of cross contamination such as having clean surfaces that may come into contact with food during processing (Lehto, Kuisma, Määttä, Kymäläinen & Mäki, 2011).Cross contamination is also relevant at retail during food handling and preparation through cutting surfaces, knives and hands (Christison, Lindsay & Von Holy, 2008; Prechter, Betz, Cerny, Wegener & Windeisen, 2002; Todd, Greig, Bartleson & Michaels, 2009). In relation with leafy green vegetables, numerous studies have focused on quantifying bacterial transfer in different contexts and situations (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003; Montville, Chen & Schaffner, 2001; Ravishankar, Zhu & Jaroni, 2010; Wachtel, & Charkowski, 2002; Wachtel, McEvoy, Luo, Williams-Campbell & Solomon, 2003). However, there is few available data on how enteric pathogens such as *E. coli* O157:H7 and *Salmonella* spp. can survive on stainless steel surfaces in presence of vegetable residues generated during processing or handling at industrial and retail levels.

Predictive models describing pathogen survival on surfaces could help to derive more accurate estimates of the probability of cross contamination under specific conditions (Pérez-Ródriguez et al., 2011, Spector & Kenyon, 2012). At present, there are limited predictive models based on the survival ability of pathogenic bacteria in food stainless steel surfaces (Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, 2013; Vogel, Hansen, Mordhorst & Gram, 2010).

Therefore, the objectives of this work were i) to evaluate survival of *Escherichia coli* O157:H7 and *Salmonella* spp. on stainless steel surface soiled with different vegetable juices (spinach, cabbage, spinach, iceberg lettuce, romaine lettuce and parsley); ii) to establish possible differences on survival capacity between vegetable juices and microorganisms and iii) based on observations, to assess suitable mathematical models (log-linear, log-linear+tail, weibull, weibull+tail and biphasic) in order to describes survival pattern for each vegetable juice and microorganism.

Materials and methods

Inoculum preparation

Two bacterial strain cocktails were prepared, consisting of five strains of *Salmonella enterica* subsp. *enterica* (CECT: 443, 4594 -*S.* Typhimurium-; and 556, 4300, 4396 -*S.* Enteritidis-) and five strains of *E. coli* O157:H7 (CECT: 4076, 4267, 4782, 4783, 5947), respectively. The bacterial strains were obtained lyophilized from the Spanish Collection of Types Culture (CECT), and reconstituted following the provider's instructions. After reconstitution, the strains were stored in beads, submerged in

cryopreservatives in commercial cryovials (Microbank^{TM}, Pro-Lab Diagnostics, USA) at -20ºC. For experiments, first a bead of each strain was transferred to a tube containing 4.5 mL Tryptone Soya Broth (TSB, Oxoid, UK) and was incubated during 24 hours, at 37 ºC. Then, three consecutive passes of the strains were made by transferring 0.5 mL of fresh bacterial culture to tubes containing 4.5 mL of TSB. After the third pass, the tube was incubated at 37ºC, for 18 and 20 h for *E. coli* O157:H7 and *Salmonella* spp., respectively. To obtain a cocktail, 1 mL of each one of the 5 strains of each microorganism (*E. coli* O157:H7 and *Salmonella* spp.) were mixed in a sterile tube, the mixture reaching a final concentration of $5x10^9$cfu/mL, approximately. Subsequently, we proceeded to remove the growth broth (TSB) through three consecutive washing steps by centrifugation at 4100 rpm for 10 min, and re-suspending, the first two times, in 5 mL of Phosphate Buffer Solution (PBS, Oxoid, UK), and, the third time, in 5 mL of sterile vegetable juice . Finally, the concentration in sterile vegetable juice obtained after this process was $1x10^9$ - $5x10^9$ cfu/mL, approximately.

Preparation of substrates based on sterile vegetable juices

Six different leafy vegetables, corresponding to spinach, cabbage, spinach, iceberg lettuce, romaine lettuce and parsley were used to elaborate different vegetable juices to be used as substrate in the survival experiments on stainless steel surfaces. For that, first, vegetables were purchased in a local market (Cordoba, Spain) and transported to laboratory under refrigeration condition (4-6 ºC). Upon arrival, a portion of 66.8 g of each vegetable were aseptically collected and placed in a stomacher bag with 100 mL sterile distilled water. The mixture was homogenized for 2 min at full power in the equipment Stomacher (IUL Instruments, Barcelona, Spain). The obtained juice was passed through successive bacteriological filters to achieve sterilization by filtration (through 1.8, 1.2, 0.8, 0.45, 0.22 µm filter, Millipore, USA) and then was kept for 7 days at -20 °C, to maintain the sterility conditions of vegetable juice.

Inoculation of stainless steel surfaces

Stainless steel coupons were delimited with 17 areas of 2x5 cm (10 cm²) each and then, by following the protocol used by Kusumaningrum, van Putten, Rombouts & Beumer, (2002), the coupons were sterilized at 121 ºC for 15 min in an autoclave. For inoculation, 0.1 ml of vegetable juice with ~10^9 cfu/mL of the test microorganism was added onto the surface and evenly distributed on the 10 cm² surfaces with the aid of the tip of a micropipette, reaching a final concentration on surfaces of 10^7-10^8 cfu/cm², approximately. This methodology, applied before by Pérez-Rodriguez et al. 2008), was repeated for all the used substrates: the six different vegetable juices and Saline Solution (0.85% NaCL). A relatively high inoculum level was preferred in order to capture the whole survival patterns of both microorganisms in the different substrates, which facilitated comparisons between different conditions with different survival capacities.

Storage and sampling of inoculated stainless steel surfaces

The sampling of the inoculated surfaces was performed using swabs of calcium alginate's tips (Calgiswab ®, Puritan Medical Product LLC., USA) at different times starting from minute 0, following the procedure of Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, (2013). The tip of the swab used to sample each surface was placed in a tube with peptone water (PA, Oxoid, UK) according to the procedure described by Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, (2013). By starting the sampling from minute 0 allowed us to establish the

capacity of recovery of the swabs used. The samples collected at the first eight times of collection (0'; 10'; 30'; 60'; 120'; 240'; 6h; 24h) were re-suspended in 5 mL of peptone water (1%) and the samples collected at the subsequent times were re-suspended in 3 mL of peptone water (1%) (36h; 48h; 72h; 96h; 120h; 144h; 168h; 192h). End time in this study corresponded to the last analysis time in which counts were obtained (i.e. above limit of quantification). Therefore, this should not be considered the end of microorganism survival, but rather, maximum time that microorganism is able to be detected or recovered from surface in the experiment. Inoculated stainless steel coupons were stored under refrigeration at around 6.5 °C and relative humidity controlled at 60-70 %, and withdrawn at proper intervals according to times mentioned above. These values of temperature and the relative humidity were chosen because they are the ones given in the white area of these industries.

Microbiological analysis

After collecting the samples with sterile alginate swabs, swab tips were placed in tubes with peptone water (1 %) as described above, and then vortexed for 1 min in order to release cells from the swab tip to peptone water according to procedure followed by Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, (2013). Then, suspensions were decimally diluted on 0.85 % saline solution and plated onto the corresponding selective agar media by using the equipment Spiral Plater (Eddy Jet, IUL, SA). Furthermore, 1 mL of the samples recovered on the last collection times was pour-plated to increase the level of detection. Therefore, the limit of quantification in this study was theoretically established to -0.52 log cfu/cm^2. The selective agar media used for both test microorganisms corresponded to Xylose-Lysine-Desoxycholate Agar (XLD, Oxoid, UK) for *Salmonella* spp. and MacConkey sorbitol Agar (SMACK, Oxoid, UK) for *E. coli* O157:H7. After incubation at 37 °C for 24 h, the number of characteristics colonies grown on the inoculated agar plates was determined.

Sterility Testing

In order to assess the sterility of filtered vegetable juices, aliquots of each were pour plated into Plate Count Agar (PCA, Oxoid, UK) and incubated at 37 °C for 24h. Likewise, a negative control sample (i.e. non-inoculated substrate) was analyzed at time 0 and end time on stainless steel surfaces in order to confirm sterility conditions of the stainless steel surfaces before and during experiments. The sampling procedure and microbiological analyses used for sterility tests on surfaces were the same as those used for inoculated area, using as selective agar media XLS and SMACK for *Salmonella* spp. and *E. coli* O157:H7, respectively, in addition to PCA as nutritive agar.

Statistical analysis and data modelling

The combination of each substrate and microorganism was repeated three times in independent experiments in order to capture biological variability. Mean counts of *E. coli* O157:H7 and *Salmonella* spp. expressed in log cfu /cm^2 were plotted with respect sampling time (h) by using Excel software (Redmond, Microsoft Corporation) and then were analyzed to assess different inactivation kinetic models.

Table 1. Description of the survival kinetic models used to analyze the experimental data obtained in this study.

Model Name	Model	Model Parameters	References
Log linear	$\log_{10} N = \log_{10} N0 * \exp(-k_{max} * t)$	k_{max}	Bigelow and Esty, 1920
Log linear + tail	$\log_{10} N = (\log_{10} N0 - \log_{10} N\_res) * \exp(-k_{max} * t) + \log_{10} N\_res$	k_{max}, N_res	Geeraerd et al., 2000
Weibull	$\log_{10} N = \log_{10} N0 - ((t/\ delta)^{\wedge}\ p))$	$delta$, p	Mafart et al., 2002
Weibull + tail	$\log_{10} N = \log_{10} [(N0 - N\_res) * 10^{\wedge}(-((t/\ delta)^{\wedge}\ p)) + N\_res]$	$delta$, p, N_res	Albert and Mafart, 2005
Biphasic model	$\log_{10} N = (\log_{10} N0 * (f * \exp(-k_{max}1 * t) + (1 - f) * \exp(-k_{max}2 * t)$	f, $k_{max}1$, $k_{max}2$	Cerf, 1977

k_{max}: the specific inactivation rate; N: the microbial population at time t; $N0$: the microbial population at time zero; N_res: the residual population density; $delta$: the scale parameter; p: the shape parameter; $k_{max}1$ and $k_{max}2$: the specific inactivation rates of the two subpopulations; f: the fraction of a major less-resistant subpopulation in the total initial population.

The Log-linear model, Log-linear with tail model, Weibull model, Weibull with tail model and Biphasic model were fitted to mean counts (log cfu/cm^2) vs. time (h) by using the curve fitting toolbox provided by the Excel Add-in, GInaFiT (Geeraerd, Valdramidis & Van Impe, 2005). The statistical indexes, RMSE (Root Mean Square Error), R^2 and adjusted R^2 (adj-R^2), at a significance level of $P \leq 0.05$, were analyzed to determine the goodness of fit of models to survival data. The correlation coefficient (R^2) measures the fraction of variation over the mean that is explained by a model. The higher the value ($0 < R^2 < 1$), the better the prediction by the model is (Montville, Chen & Schaffner, 2001; Jin, Zhang, Hermawan & Dantzer, 2009). Adj-R^2 is a modification of R^2 that adjusts for the number of explanatory terms in a model (i.e. residual degrees of freedom). On the other hand, a value of RMSE closer to 0 indicates a better fit (Ratkowsky, 2002). By comparison of the values of these indexes obtained from each model we could determine how better the model predicts the experimental survival data. In addition, the corrected Akaike´s Information Criterion (AICc) was used to enable comparison between different (i.e. non-nested) models based on goodness of fit to data, number of regression parameters (i.e. parsimony principle), and number of samples (n). According to AICc, the best model is the one with the lowest AICc value.

Results and Discussion

Recovery capacity

The swabbing method was preferred instead others (e.g. impression methods) because it allows to better remove organic matter (soil conditions) where cells are embedded, thereby enabling better recovery of cells (Pérez-Rodríguez, Valero, Carrasco, Garcia-Gimeno & Zurera, 2008). Results obtained at time 0 (wet conditions) and at 2 hours (dry conditions) were analyzed in order to assess the recovery capacity of the method used for sampling inoculated surfaces. The recovery rates were calculated as percentages of recovered cells with respect to the initial inoculum deposited on the surfaces. For *E. coli* O157:H7, mean recovery rates ranged between 19 and 46%, with SS and parsley showing the lowest and highest values, respectively. These values coincided with results (29-45 %) obtained from a similar work carried out in our laboratory with the same sampling method in SS, TSB and meat purge (Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, 2013). In other similar study, the swabbing method was able to recover 2-10 % *E. coli* inoculated on stainless steel coupons using water-based solutions with different organic loads (Martinon, Cronin, Quealy, Stapleton & Wilkinson, 2012). Recovery rates for *Salmonella* spp. were lower, ranging 3 and 32 %, with the lowest value in SS, and the highest recovery percentage, in Iceberg lettuce. In dry conditions, recovery rates showed a huge decrease as compared to wet conditions, with values ≤ 0.3 % for both microorganisms in the different substrates. In this case, the desiccation or dehydration process undergone by cells could affect cell viabilities, thereby reducing recovery rates. Results from a previous study in our laboratory (Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, 2013) demonstrated higher recovery rates for SS with *E. coli* O157:H7 (1.6 %); however, in that study, dry conditions were observed at 1 h after inoculation due to a lower relative humidity. A study by Foschino, Picozzi, Civardi, Bandini & Faroldi, (2003) also reported low recovery percentages for *E. coli* in different stainless steel surfaces, at dry conditions, with percentages ranging from 0 to 4 %. Similar results were obtained by Martinon, Cronin, Quealy, Stapleton & Wilkinson, (2012) for *E. coli* after 1-h incubation on stainless steel coupons, with recovery

Table 2. Summary of survival data for *Escherichia coli* O157:H7 obtained in stainless steel surfaces soiled with different vegetable juice substrates

Substrate	S_i	S_f	ΔS_{2h}	ΔS_{24h}	ΔS_{72h}	ΔS_f	End time
Chard	7.98 ± 0.23	1.88 ± 0.72	3.00 ± 0.61^{ab}	3.93 ± 0.48a^{b}	4.60 ± 0.43	6.22 ± 0.72	192h
Red cabbage	7.64 ± 0.58	2.40 ± 0.23	2.05 ± 0.07^{a}	3.28 ± 0.42^{a}	3.77 ± 0.19	5.19 ± 0.23	192h
Spinach	8.09 ± 0.58	1.62 ± 0.2	2.81 ± 0.54 ^{ab}	6.17 ± 0.37^{d}	ND	6.47 ± 0.20	32h
Iceberg lettuce	8.18 ± 0.07	1.89 ± 0.83	3.57 ± 0.96^{ab}	4.43 ± 0.80^{abc}	5.58 ± 0.53	6.30 ± 0.83	192h
Parsley	8.43 ± 0.16	0.30 ± 0.51	4.54 ± 0.94^{b}	6.58 ± 0.60^{d}	ND	7.90 ± 0.51	48h
Romaine lettuce	8.44 ± 0.23	0.20 ± 0.38	2.69 ± 0.61^{ab}	5.63 ± 0.80^{cd}	6.06 ± 0.26	8.24 ± 0.38	144h
Saline Solution	7.74 ± 0.50	0.59 ± 0.09	3.62 ± 0.39^{ab}	5.15 ± 0.30^{bcd}	6.35 ± 0.44	7.14 ± 0.09	120h

S_i , S_f correspond to concentrations (log cfu/cm²) observed at time 0 h and end time, respectively.
ΔS_{2h} , ΔS_{24h} , ΔS_{72h} , ΔS_f correspond to logarithmic reductions (log cfu/cm²) observed at different times.
Letters (a, b, c, d) in each column show the different homogenous groups reported by Tukey´s HSD test ($P \leq 0.05$).
ND: Not Determined

Table 3. Summary of survival data for Salmonella spp. obtained in stainless steel surfaces soiled with different vegetable juice substrates

Substrate	S_i	S_f	ΔS_{2h}	ΔS_{24h}	ΔS_{72h}	ΔS_f	End time
Chard	7.68 ± 0.51	0.87 ± 0.43	1.96 ± 0.17^{ab}	3.58 ± 0.27^{a}	4.88 ± 0.41	6.26 ± 0.43	168h
Red cabbage	7.74 ± 0.53	0.73 ± 0.90	1.78 ± 0.69^{a}	3.70 ± 0.55^{a}	4.44 ± 0.52	6.52 ± 0.90	168h
Spinach	7.80 ± 0.88	0.89 ± 0.15	3.02 ± 0.35^{ab}	6.05 ± 0.45^{b}	ND	6.53 ± 0.15	48h
Iceberg lettuce	7.76 ± 0.39	1.48 ± 0.89	2.58 ± 0.18^{ab}	3.80 ± 0.27^{a}	4.91 ± 0.33	6.46 ± 0.89	168h
Parsley	7.47 ± 0.37	0.86 ± 0.08	3.23 ± 0.96^{b}	5.20 ± 0.57^{ab}	ND	6.61 ± 0.08	48h
Romaine lettuce	7.57 ± 0.70	0.68 ± 0.34	2.16 ± 0.54^{ab}	4.04 ± 0.48^{ab}	5.72 ± 0.11	7.68 ± 0.34	168h
Saline Solution	7.04 ± 0.14	0.90 ± 0.27	2.41 ± 0.24^{ab}	4.36 ± 0.27^{ab}	5.43 ± 0.39	6.14 ± 0.27	120h

S_i , S_f correspond to concentrations (log cfu/cm²) observed at time 0 h and end time, respectively.
ΔS_{2h} , ΔS_{24h} , ΔS_{72h} , ΔS_f correspond to logarithmic reductions (log cfu/cm²) observed at different times.
Letters (a, b, c, d) in each column show the different homogenous groups reported by Tukey´s HSD test ($P \leq 0.05$).
ND: Not Determined

percentages of 0-2 % depending on the used organic load. Besides that, variability between experiments was reasonably acceptable since standard deviation values were around 2-30 % for wet conditions and below 0.4 % for dry conditions. These variation percentages account for values < 0.5 log cfu/cm^2, which corresponds to the plate count error. As survival is supposed to follow a logarithmic process, variability was expected not to significantly affect the observed survival patterns. In addition, the recovery method was satisfactorily applied in a previous study dealing with survival of *S. aureus* and *E. coli* O157:H7 on stainless steel surfaces (Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, 2013). Therefore, the sampling method was deemed to be valid to assess survival of both pathogens in the different substrate as a function of time.

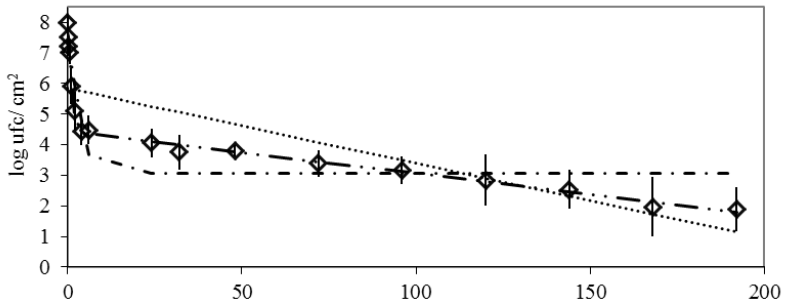
Survival pattern of *E. coli* O157:H7 and *Salmonella* spp. on stainless steel surfaces with different vegetable juice substrates

The behavior of *E. coli* O157:H7 and *Salmonella* spp. populations on stainless steel surfaces soiled with different vegetable juice substrates and SS are shown in Figure 1 and 2, respectively. The graphs in these figures plot the number of survival cells (log cfu/cm^2) with respect to time (h) for different microorganisms and substrates. In general, through visual analysis of graphs, it can be noted that the survival pattern in both microorganisms is similar and reflects a pronounced log-decrease for the first 6 hours (with a high inactivation rate) followed by a slowing-down of the inactivation rate, resulting in an apparent tailing effect, which remains up to one week (~8 days) in some substrates. None survival curves displayed shoulders. This concave upward survival pattern shown by both microorganisms suggests the existence of two bacterial populations. One comprised by more sensitive cells to hydric stress (i.e. high inactivation rate) and other by a residual bacterial population of more resistant cells to hydric stress (i.e. low inactivation rate). On the other hand, Table 2 and 3 show log-decrease values obtained on 7 substrates for both microorganisms at selected incubation times (2, 24, 72 h) and end time. These values were statistically analyzed to determine significant differences among substrates and microorganisms, except for the end time due to it was different between substrates and microorganisms. Some substrates were not sampled at time 72h and therefore they could not be compared with other substrates at that time. Overall, results indicated that type of microorganism significantly affected log-reduction values at the different analyzed times and substrates ($P \leq 0.05$). Furthermore, there were significant differences among substrates at 2 and 24 h for both microorganisms ($P \leq 0.05$).

The log-reductions of *E. coli* O157:H7 and *Salmonella* spp. at different times on stainless steel surfaces soiled with different vegetable juice substrates

In Table 2, it can be observed that at 2 h, the largest log-reduction of the microorganism was given in parsley (4.54 log cfu/cm^2), followed by a homogenous group comprised by spinach, lettuce and chard substrates as well as SS (3.62-2.69 log cfu/cm^2); and finally the lowest reduction corresponded to that obtained in red cabbage (2.05 log cfu/cm^2) ($P \leq 0.05$). At 24 h, results showed a similar pattern to that observed at 2 h, with the greatest accumulated decrease in parsley and spinach (6.17-6.58 log cfu/cm^2), followed by romaine lettuce (5.63 log cfu/cm^2), SS (5.15 log cfu/cm^2), iceberg lettuce (4.43 log cfu/cm^2), chard (3.93 log cfu/cm^2) and finally red cabbage with the lowest accumulated drop (3.28 log cfu/cm^2) ($P \leq 0.05$). Again, the behavior was similar at 72 h, with the exception that no cells were recovered in parsley and spinach, due to levels were likely below the limit of quantification (Table 2).

a)


log ufc/ cm²
0
1
2
3
4
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8
0
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100
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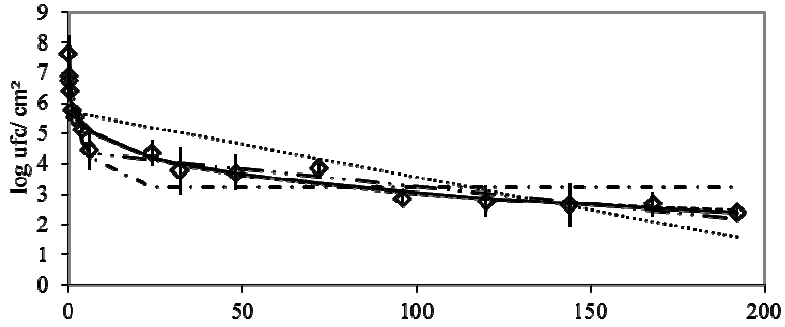
200

b)


log ufc/ cm²
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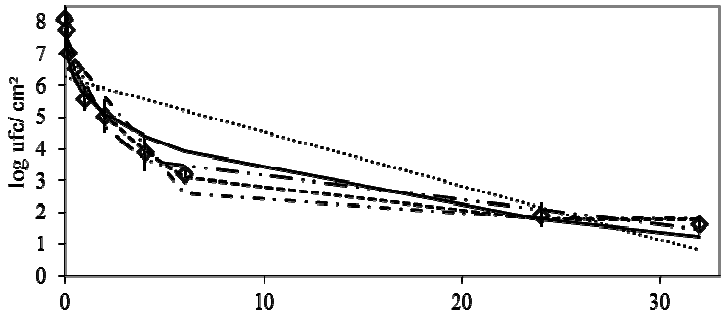
200

c)


log ufc/ cm²
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1
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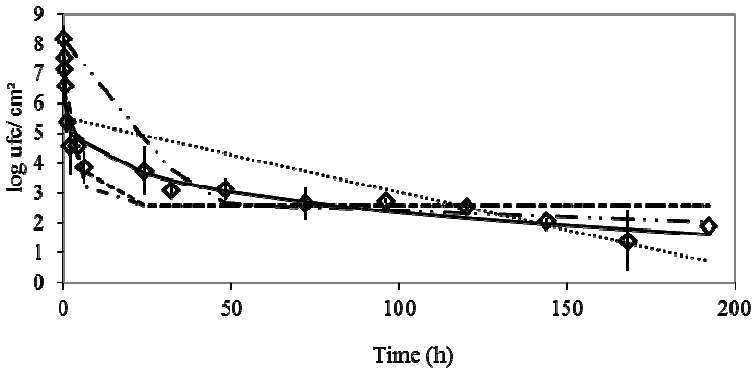
20
30

d)


log ufc/ cm²
0
1
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150
200
Time (h)

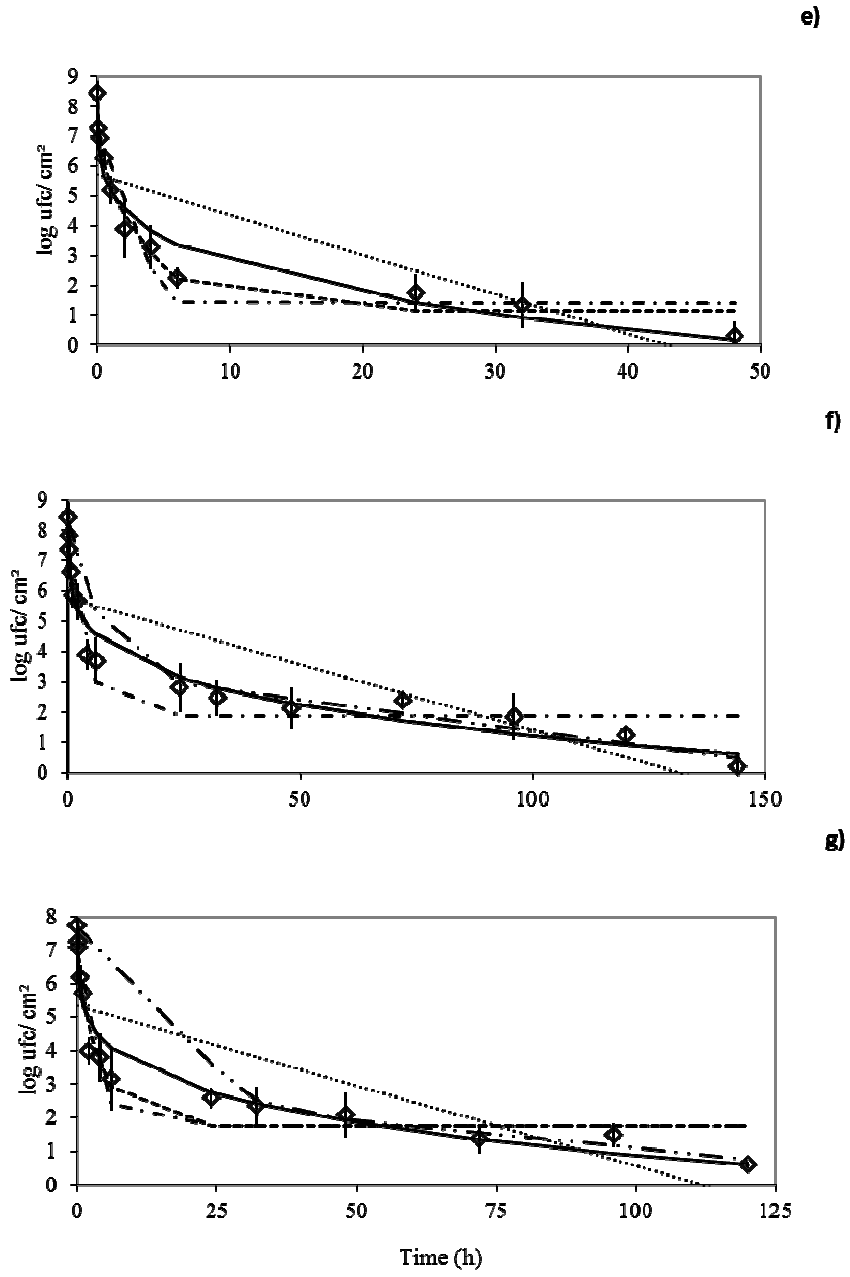
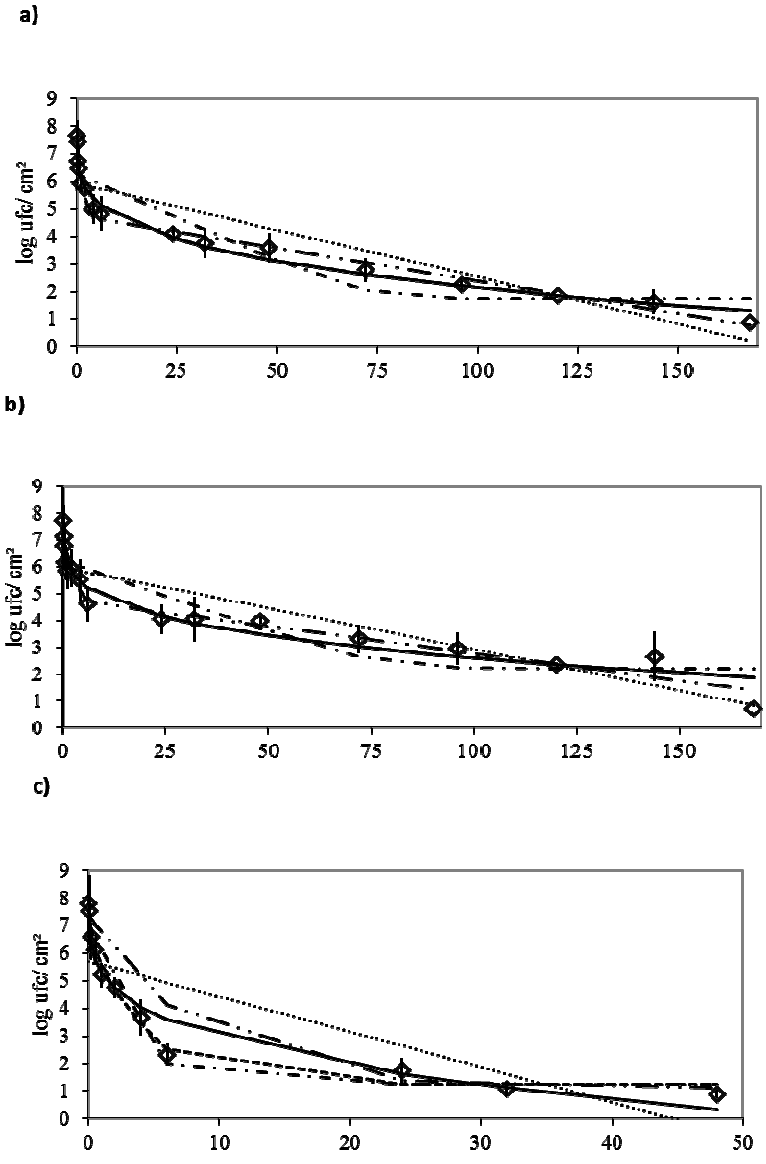
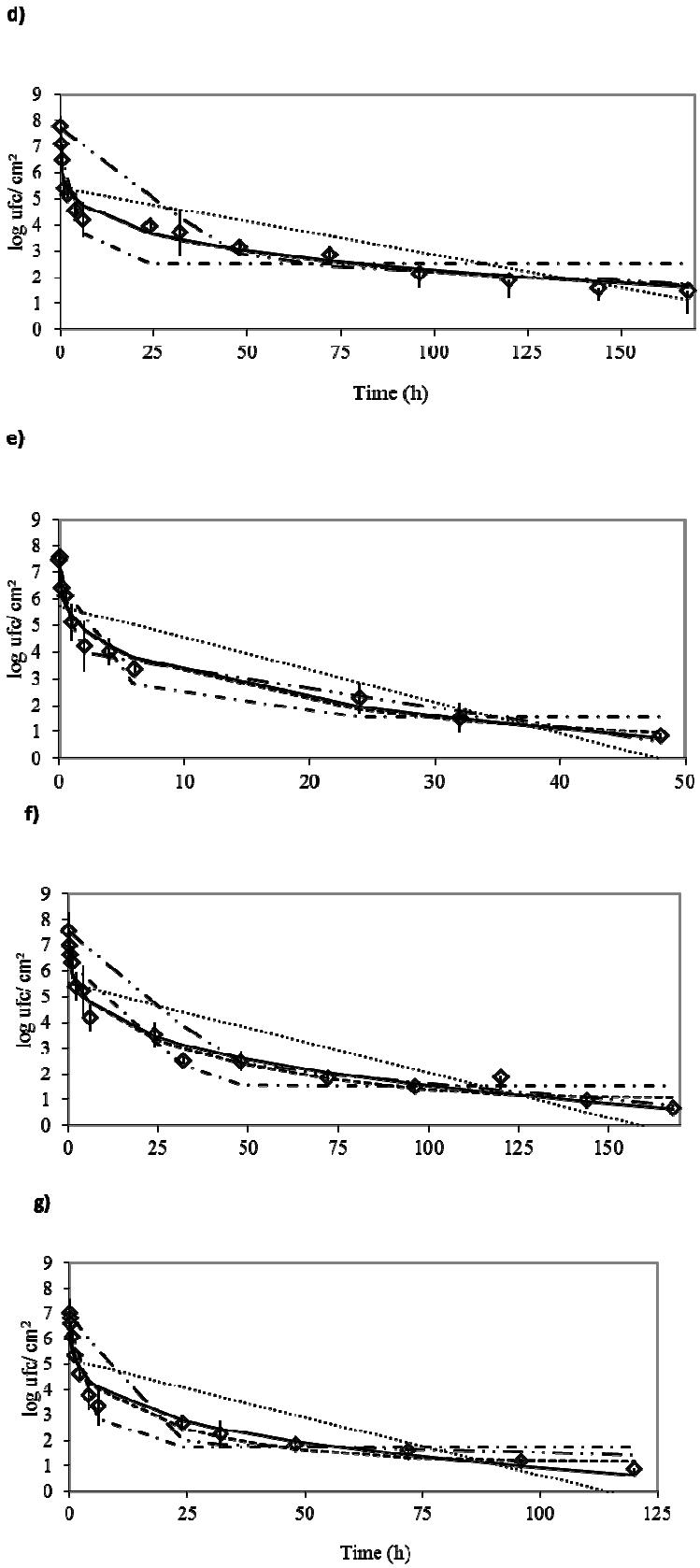


Figure 1.··· Survival data (mean and standard deviation) obtained for *Escherichia coli* O157:H7 on stainless steel soiled with chard (a), red cabbage (b), spinach (c), iceberg lettuce (d), parsley (e), romaine lettuce (f) and saline solution (g), and fitted to log-linear model (·····), log-linear with tail model (··_ _ . _ _·), Weibull model (·····), Weibull with tail model (- - - - - -) and biphasic model (·· — ·· — ···).¶

Regarding end time, the highest and lowest total log-reductions corresponded to 8.24±0.38 and 5.19±0.23 log cfu/cm^2 for romaine lettuce (144h) and red cabbage (192h) respectively. For substrates with 192 h as end time, that is, chard, red cabbage and iceberg lettuce, total log-reductions were 6.22, 5.19 and 6.30 log cfu/cm^2, respectively. The shortest survival time corresponded to spinach y and parsley with end times of 32h and 48h and total reductions of 6.47 y 7.90 log cfu/cm^2, respectively. Results for *Salmonella* spp. was in keeping with survival patterns found for *E. coli* O157:H7 shown above. Again, substrate affected the accumulated log-decrease at times 2 and 24 h,

obtaining three homogeneous groups ($P \leq 0.05$), with similarities between times, even though some slight difference were found as shown in Table 3. The log-decrease values obtained at 2 h showed that the highest drop occurred in parsley (3.23 log cfu/cm^2), followed by a homogenous group (3.02-1.96 log cfu/cm^2) with spinach, iceberg lettuce, SS, romaine lettuce and chard ($P \leq 0.05$) . The smallest reduction at 2 h was found in red cabbage (1.78 log cfu/cm^2) ($P \leq 0.05$). The survival pattern slightly changed at 24 h. In this case, the greatest accumulated log-decrease was found for spinach (6.05 log cfu/cm^2) ($P \leq 0.05$); however, the next in reduction corresponded to parsley substrate included in a homogenous group with SS, romaine and iceberg lettuce (5.20-4.04 log cfu/cm^2) ($P \leq 0.05$). The substrates with the lowest log-decrease values were red cabbage and chard (3.70 and 3.58 log cfu/cm^2, respectively) which formed a homogenous group, according to the statistical test ($P \leq 0.05$). This pattern was also observed for the accumulated log-decrease at 72 h with the lowest drop observed in red cabbage. Alike *E. coli* O157:H7, no counts were obtained for parsley and spinach, indicating that cell levels on surface at 48 h were below the limit of quantification. Looking at the end time, spinach and parsley showed the shortest survival time which corresponded to 48h, followed by SS with 120 h and the rest of substrate with an end time of 168 h.





Figure·2.···Survival·data·(mean·and·standard·deviation)·obtained·for·*Salmonella*·spp.·on·stainless·steel·soiled·with·chard·(a),·red·cabbage·(b),·spinach·(c),·iceberg·lettuce·(d),·parsley·(e),·romaine·lettuce·(f)·and·saline·solution·(g),·and·fitted·to·log-linear·model·(···.....···),·log-linear·with·tail·model·(··_ _ _ _·),·Weibull·model·(————),Weibull· with· tail· model(· - - - - - - -)·and·biphasic·model·(· —··—··—).¶

Comparing both microorganisms of *E. coli* O157:H7 and *Samonella* spp. on stainless steel surfaces soiled with different vegetable juice substrates.

In general, log-decrease values at 2, 24 and 72 h obtained in *Salmonella* spp. were lower than those for *E. coli* O157:H7 with some exceptions such as red cabbage in which the drop was slightly greater in *Salmonella* spp. Moreover, parsley and spinach, the substrates less supporting bacterial survival, allowed a longer *Salmonella* spp. survival (i.e. longer end time). Due to the most substrates showed this similar trend, it could be hinted that *Salmonella* spp. in this study were more tolerant to hydric stress than *E. coli* O157:H7. The differences were more evident at 2 h in which the desiccation process could have exerted a major damage on cells and therefore, a major log-decrease in the case of *E. coli* O157:H7, the most susceptible microorganism according to our results.

The first two hours played a crucial role on the survival of pathogen because of the desiccation process undergone by cells. The variations in the desiccation process could lead to the different survival patterns observed in each substrate, probably depending on the solute concentration (Møretrø et al., 2010). Also, the mechanisms of adaptation of the microorganism to the desiccation conditions in the different substrates could affect its survival capacity (Møretrø et al., 2010; Vogel, Hansen, Mordhorst & Gram, 2010). Nonetheless, the presence of antimicrobial substances cannot be precluded as other possible cause in spite of the short exposure period (2h). These results indicate that the type of microorganism and its specific metabolic and physiologic characteristics are determinant in their resistance to desiccation process.

Comparing with other studies, the log-reductions of *E. coli* O157:H7 and *Samonella* spp. on stainless steel surfaces.

For *E. coli* O157:H7, the overall log-reductions observed in our study on vegetable juice substrates were generally higher (> 6 log cfu) than those reported by other studies for E. coli (Bae, Baek & Lee, 2012; Bale, Bennett, Beringer & Hinton, 1993; Møretrø et al. 2010; Wilks, Michels & Keevil, 2005). In a previous study carried out in our laboratory (Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, 2013), E. coli O157:H7 could be recovered during longer time (200 h) on stainless steel surfaces with meat purge substrate, giving rise to an overall log-reduction equal to 4 log cfu/cm^2. However, different initial levels are expected to affect overall log-reduction, and therefore, results are not fully comparable in those cases. Looking at specific times, in the case of the study by Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, (2013), the same cocktail of E. coli O157:H7 strains showed 2.05 and 1.55 log-reductions at 2 h for SS and meat purge, respectively while in the present study, all substrates including SS resulted in reductions higher than 2 log cfu/cm^2 excepting for red cabbage, which showed a drop of 2.05 log cfu/cm^2 (Table 2). The experiments developed by Wilks, Michels & Keevil, (2005) dealing with E. coli O157 in TSB inoculated on stainless steel, and incubated at refrigeration temperatures, showed that the microorganism fell by 1 log cfu in the first three hours. Again, results indicated a lower decay as compared to our results, in addition to a longer survival time (672 h). Møretrø et al. 2010) assessed different Shigatoxin-producing E. coli strains on stainless steel surfaces at 70 % RH and 12 ºC. Their results evidenced a log-reduction of 1.5-3.5 log cfu after 24 hour, being lower than ours at the same incubation time, which ranged between 3.28 and 6.58 log cfu/cm^2. Red cabbage was again the vegetable substrate showing a more similar log-reduction with a drop of 3.28 log cfu/cm^2.

In the case of *Salmonella* spp., log-reductions were generally greater than those observed by other works (Allan J. T., Yan Z., & Kornacki J. L., 2004; Bae, Baek &

Lee, 2012; de Cesare, Sheldon, Smith & Jaykus, 2003; Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003; Møretrø et al., 2010). However, some exceptions to this fact were found in literature, as for example in the study by Castelijn, Parabirsing, Zwietering, Moezelaar & Abee (2013), which reported log-reductions of 6-7 log cfu after 5 days (120 h) for different serogroups of Salmonella inoculated on stainless steel surfaces with growth culture broth. In this study, experimental conditions corresponded to 25 ºC and a wide RH range (i.e. 35-65 %). These more stringent and variable environmental conditions would explain such greater reduction rates, since in these circumstances; desiccation process is expected to be more intense, increasing cell damage. Likewise, the study by de Cesare, Sheldon, Smith & Jaykus, (2003) showed that Salmonella spp. populations declined 5-6 log cfu after ~24 h when inoculated with PBS on stainless steel surfaces. This value was higher than those obtained in our study for different vegetable juice substrates, and even for SS (Table 3). Apart from the probable differences derived from the use of different Salmonella species, a much higher temperature (27 ºC) and lower RH (60 %) could be factors also increasing the effect of the desiccation process, thereby reducing cell viability in the study by de Cesare, Sheldon, Smith & Jaykus, (2003). However, when in this study, bacteria were inoculated with culture broth (a more protective substrate), reductions were around 3 log cfu (24h), which were lower than those obtained in our experiment at the same time (Table 3). In keeping with this, Kusumaningrum, Riboldi, Hazeleger, & Beumer, (2003) reported that Salmonella Enteritidis inoculated with SS on stainless steel surfaces declined up to 3.5 and 4.4 log cfu after 24 and 96 h, respectively (at 20-25 ºC and 40-45 % RH). These values are only comparable to those obtained for chard and red cabbage, with drops of 3.58 and 3.70 log cfu/cm^2 (24 h), respectively (Table 3) and even SS in our study resulted in higher log-reduction (4.36 log cfu, after 24 h). When organic residues (chicken and milk) were used instead of SS, observed log-reductions were much lower (data not shown by authors), indicating in this case, organic matter enhances survival capacity.

As evidenced above, differences between experiments make difficult to draw clear conclusions because variations in incubation temperature, RH, substrate, air-drying conditions, strain, initial inoculum, experiment durations and recovery method could affect results (Fuster-Valls, Hernández-Herrero, Marín-de-Mateo & Rodríguez-Jerez, 2008; Møretrø et al., 2010). Nonetheless, overall results from our study seem to demonstrate a more a rapid log-reduction than those reported in other previous studies. Besides that, in most studies discussed inhere; the use of organic substrates (i.e. food sera, culture broth, etc.) resulted in an increase in survival capacity of bacteria (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003; Møretrø et al. 2010). However, this fact can not be seen clearly in our results, in which some vegetable juice substrates ,particularly spinach and parsley, showed much greater log-reductions (at different times) than that obtained in SS (i.e. substrate simulating cleaning conditions).

Parsley and spinach juice substrates reduce the survival capacity of *E. coli* O157:H7 and *Samonella* spp. on stainless steel surfaces

Parsley and spinach were the substrates that most reduced the survival capacity of both microorganisms, with end times lower than 48 hours. This result become specially relevant when compared to SS (simulating cleaning conditions in this study), which showed an end time of 120 h and lower log-reduction values than those showed by the vegetable juice substrates at 2, 24 and 72 h. Note that SS is only formulated with NaCl (0.85 %) and water, and no organic matter is in. In turn, vegetable juices are supposed to contain a high load of organic matter released from vegetable cells during the

homogenization process. As mentioned by Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, (2013) soiled surfaces are expected to enhance survival ability, which has been also observed by other authors for Verotoxin-producing *E. coli* and *Salmonella* spp. on surfaces soiled with artificial culture media containing protein and glucose (de Cesare, Sheldon, Smith & Jaykus, 2003; Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003; Møretrø et al. 2010). This fact has been also demonstrated for *Staphylococcus aureus* on food sera such as pasteurized skimmed milk (Oulahal, Brice, Martial & Degraeve, 2008) or dried mussel-cooking juices (Herrera, Caboa, González, Pazos & Pastoriza, 2007). However, results in our study suggest that certain vegetable juices exerted a remarkable negative effect on the survival ability of both enteric pathogens. In this sense, there are also studies reporting that food sera reduces bacterial survival capacity on stainless steel surfaces, such as pork and salmon sera for *Salmonella* spp or *Listeria monocytogenes,* respectively (Allan J. T., Yan Z., & Kornacki J. L. 2004; Vogel, Hansen, Mordhorst & Gram, 2010) In our study, as possible explanation, we suggest the existence of compounds in the vegetables that once released because of the juice elaboration process would be able to exert any kind of antimicrobial effect on test microorganisms inoculated on the surface (Nutt, Li, Woodward, Zabala-Díaz & Ricke, 2003;Tajkarimi, Ibrahim & Cliver, 2010). Indeed, certain edible vegetable extracts have demonstrated to have antimicrobial activity against pathogenic bacteria (Kim et al. 2013). The study by Wong & Kitts (2006), reported that parsley extracts obtained in water and methanol gave rise to cell damage in *E. coli*, suggesting that polyphenolic compounds could impair the function and integrity of cell membrane as suggested by other authors (Farag, Daw & Abo-Raya, 1989; Raccach, 1984). In the case of spinach, no studies were found dealing with its antimicrobial properties; however, given its high polyphenol content (Pandjaitan, Howard, Morelock & Gil, 2005); it is reasonable to think that this substrate could reduce cell viability as evidenced in our results. Although in less extent, our observations indicated that also lettuce juice substrates could reduce bacterial survival on surfaces. This would be supported by some studies reporting, for lettuce, high polyphenol contents and an attendant antibacterial activity (Edziri et al., 2011). Other antimicrobial compounds released from vegetable cells because of homogenization process could be affecting bacterial cell viability in our study. Among them, we highlight specific peptides, saponins, glucosides, alkaloids, organic acids and others that are part of the original plant defense system against microbial infections (Bajpai, Rahman & Kang, 2008). Nonetheless, this hypothesis has not been proved and it would be very helpful for future investigations, to identify and characterize, biochemically and enzymatically, those compounds responsible for reducing survival capacity on surfaces of these pathogens and others.

Survival models for *E. coli* O157:H7 and *Samonella* spp. on stainless steel surfaces soiled with different vegetable juice substrates

Survival data were used to fit different mathematical models: Log-linear model; log-linear + tail model; the Weibull model; Weibull + tail model and the biphasic model. These models are also represented in Figure 1 and 2 for *E. coli* O157:H7 and *Salmonella* spp. The goodness-of-fit indexes obtained from the regression analysis for each condition and type of model are shown in Table 4 and 5, for *E. coli* O157:H7 and *Samonella* spp., respectively. In general, the model with the best fitting to survival data was the Weibull model with adj-$R^2 \geq 0.94$. Likewise, the Akaike Information Criterion corrected (AIC_c) also corroborated the Weibull model as the best choice in most cases, since this index allows to compare non-nested models with different number of

regression parameters (e.g. log-linear model and Weibull model). The lowest value of AIC_c indicates the best model, which means better fitting with less regression parameters (i.e. parsimony principle). Nonetheless, the Weibull+tail model showed better indexes in some cases, which will be commented below. On the contrary, the log-linear models generally resulted in worse fittings, with worse values for the goodness-of-fit indexes as shown in Table 4 and 5, for *E. coli* O157 and *Salmonella* spp., respectively. This worse fitting for the log-linear model was expected given the concave upward pattern shown by survival curves of both microorganisms in the different substrates. More specifically, *E. coli* O157:H7 survival in substrates of spinach, parsley and SS were better described by the Weibull + tail model with adj-R^2 = 0.99, 0.97, 0.94; and AICc = -37.23, -29.00 and -38.56, respectively. In turn, the Weibull model was more suitable for the substrates of red cabbage, iceberg lettuce and romaine lettuce with adj-R^2= 0.97, 0.94, and 0.95; and AICc= -84.03, -62.00, and -47.63, respectively. Finally, the biphasic model presented better fitting to survival data in chard (adj-$R^2 \geq 0.99$; AICc= -95.80). Both adj-R^2 and AICc obtained the best scores for the models with the best fitting in all substrates excepting for SS in which the lowest value of AICc (-41.07) corresponded to the Weibull model while the highest value of adj-R^2 was obtained for the Weibull-tail model (Table 4).With regard to *Salmonella* spp. again the Weibull model was the best choice; with chard, red cabbage, iceberg lettuce, romaine lettuce and saline solution showing adj-R^2= 0.98, 0.95, 0.96, 0.97, and 0.96; and AICc= -83.8, 66.94, -72.84, -60.24, and -50.31, respectively. In contrast, *Salmonella* spp. survival in spinach and parsley was better represented by the Weibull + tail model and biphasic model, respectively. Alike *E. coli* O157:H7, the highest scores of adj-R^2 and the lowest values of AICc were always obtained in the same model, corroborating the choice of the best model in all cases (Table 5).

In Table 6 and 7, regression parameters for the fitted models are reported for *E. coli* O157:H7 and *Salmonella* spp. The kinetic parameters (i.e. inactivation rates) obtained in the best-fit models were used to assess the survival capacity in each substrate. In the case of the Weibull model, the interpretation of parameters *delta* and *p* is difficult due to both jointly define the survival curve. In a previous work (Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, 2013), it was indicated that *delta* can be seen as a non-linear inactivation rate representing the overall steepness of the survival curve when *p* is fixed (Peleg, M., 2006). For different *p* values, we could consider the exponential effect of *p* on *delta* as a valid mathematical criterion to assess survival capacity (i.e. inactivation rate) (Table 1), which could be represented by the power $delta^p$. If the inverse is made, the resultant expression, i.e. $1/(delta^p)$, is equivalent to the term *b* in the power law version of the Weibull model ($Log_{10}(N/N0) = -bt^n$), which is also considered as a nonlinear rate parameter (Peleg, M., 2006). This mathematical term means that the lower value from $delta^p$, the higher the inactivation rate. Therefore, on the basis of this criterion, in *E. coli* O157:H7, the lowest survival capacity was evidenced in parsley followed by romaine lettuce, spinach and iceberg lettuce, saline solution, chard and red cabbage. The red cabbage was the one with the lowest inactivation rate ($1/0.04^{0.20}$=1.90) while that parsley was the substrate with the highest rate ($1/0.003^{0.22}$=3.59). In the case of S*almonella* spp. the lowest survival capacity was observed in spinach and parsley ($1/0.01^{0.25}$=3.16 and $1/0.03^{0.27}$=2.58, respectively) followed by iceberg lettuce, romaine lettuce, and saline solution, while the greatest survival capacity was found in chard and red cabbage with the lowest reduction rate ($1/0.17^{0.27}$=1.61 and $1/0.18^{0.26}$=1.58, respectively). These results slightly differ from those given for *E. coli* O157:H7 although follow a similar pattern, which is also in agreement with conclusions derived from the analysis of survival data in Table 2 and 3,

in which log-decreases at different times are compared between substrates.

The use of quantitative kinetic parameters, as here described, may facilitate comparisons between different studies, microorganisms and substrates. Therefore, we made an attempt to compare our estimates for the kinetic parameters from the Weibull model to those obtained by Vogel, Hansen, Mordhorst & Gram, (2010) and Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, (2013); on *L. monocytogenes* and *E. coli* O157:H7 on stainless steel surfaces, respectively. In the former case, *delta* and *p* values greatly depended on the type of Listeria strain, initial inoculum and substrate (i.e. growth culture medium, fresh salmon juice, and smoked salmon juice). Importantly, $1/delta^p$ values (expressed in hours) obtained when surfaces were soiled with fresh and smoked salmon juice were quite high (>0.014) in comparison to our results, indicating a much better survival capacity of *L. monocytogenes* in food substrate (>90 days), even though that result is expected as the Gram + character of this microorganism, which confers it a higher resistant to hydric stress.

In the case of *E. coli* O157:H7, *delta* values obtained by Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, (2013), in SS, TSB and meat purge, were more similar to ours, while *delta* values obtained from vegetable juices substrates tended to be lower (<0.2). Indeed, in that study, the pathogen could be detected, on stainless steel surface soiled with meat purge, for a longer period (200 h) in spite of its lower initial inoculum ($\sim 10^5$ cfu/cm^2). This fact could be also evidenced by a slightly higher $delta^p$ value for meat purge substrate, which means a lower reduction rate ($1/0.76^{0.23}=1.06$). In the study by Pérez-Rodríguez, Posada-Izquierdo, Valero, Garcia-Gimeno & Zurera, (2013), *Staphylococcus aureus* was also tested. In that case, *delta* values were much higher (>10) than those obtained for the microorganisms studied herein, meaning a lower reduction rate. The well-known high resistance of *S. aureus* to hydric stress and desiccation process was the main explanation for such low reduction rate. However, *S. aureus* in that study seemed to better follow a log-linear trend denoted by $p \approx 1$ in the Weibull model.The impact of cross contamination on microbiological risk greatly depends on the number of survival cells on surfaces, i.e. viable cells able to transfer to foods and then grow up to risk levels. Incorporating survival models in quantitative risk assessment can be crucial to accurately represent cross contamination process at factory and retail. Given that survival experiments were developed under similar conditions to those occurring in IV gamma vegetable factories (6 ºC and 70 % RH), survival models could be efficiently applied to Quantitative Risk Assessment studies to simulate more realistic scenarios and provide more accurate estimates of cross contamination in fresh-cut vegetable processing lines. In such a way, by combining transfer rates, contact frequencies, initial levels on surfaces, disinfection and survival models, the number of cells transferred to foods can be estimated for any time during production. All this quantitative information is the great value to support decision-making processes, to rank risk scenarios and to prioritize control measures. In addition, as demonstrated in this section, models could be used to comparison purposes based on obtained kinetic parameters and the influence of various factors can be assessed such as type of microorganism, surface and substrate.

Table 4. Goodness-of-fit indices for both log-linear model, log-linear with tail model, Weibull model, Weibull with tail model and biphasic model fitted to survival data of *Escherichia coli* O157:H7 on stainless steel inoculated with different substrates (chard, red cabbage, spinach, iceberg lettuce, parsley, romaine lettuce and saline solution).

		Substrate						
Models	**Goodness-of-fit index**	Chard	Red cabbage	Spinach	Iceberg	Parsley	Romaine	SS*
	AICc**	-38.90	-45.52	-10.90	-33.28	-8.98	-22.20	-20.01
Log-linear	adjusted-R^2	0.66	0.69	0.65	0.59	0.60	0.64	0.60
	RMSE	1.14	0.94	1.38	1.34	1.72	1.56	1.53
	AICc	-50.26	-56.99	-22.28	-48.72	-20.32	-38.95	-31.52
Log-linear+tail	adjusted-R^2	0.85	0.87	0.93	0.86	0.90	0.90	0.90
	RMSE	0.75	0.61	0.63	0.78	0.86	0.80	0.90
	AICc	N.A.***	-84.03	-26.74	-62.00	-26.22	-47.63	-41.07
Weibull	adjusted-R^2	N.A.	0.97	0.95	0.94	0.94	0.95	0.93
	RMSE	N.A.	0.28	0.50	0.53	0.66	0.60	0.64
	AICc	N.A.	-79.44	-37.23	-53.43	-29.00	-42.51	-38.56
Weibull+tail	adjusted-R^2	N.A.	0.97	0.99	0.92	0.97	0.94	0.94
	RMSE	N.A.	0.29	0.22	0.61	0.46	0.63	0.61
	AICc	N.A.	-71.64	-23.78	-15.78	N.A.	-21.10	-4.84
Biphasic model	adjusted-R^2	0.99	0.95	0.97	0.22	N.A.	0.76	0.30
	RMSE	0.18	0.36	0.43	1.86	N.A.	1.28	2.02

* SS: Saline Solution (0.85 %)
** AICc: Akaike´s Information Criterion
***NA: Not Adequate

Table 5. Goodness-of-fit indexes for both log-linear model, log-linear with tail model, Weibull model, Weibull with tail model and biphasic model fitted to survival data of *Salmonella* on stainless steel inoculated with different substrates (chard, red cabbage, spinach, iceberg lettuce, parsley, romaine lettuce and saline solution).

		Substrate						
Models	**Goodness-of-fit index**	Chard	Red cabbage	Spinach	Iceberg	Parsley	Romaine	SS*
Log-linear	AICc**	-46.03	-47.84	-8.63	-39.73	-15.65	-29.26	-24.49
	adjusted-R^2	0.82	0.80	0.64	0.69	0.70	0.77	0.65
	RMSE	0.92	0.88	1.54	1.11	1.27	1.24	1.30
Log-linear+tail	AICc	-47.59	-44.66	-23.10	-45.81	-22.14	-40.23	-43.98
	adjusted-R^2	0.86	0.80	0.94	0.82	0.84	0.89	0.93
	RMSE	0.81	0.88	0.60	0.85	0.79	0.77	0.58
Weibull	AICc	-83.38	-66.94	-22.20	-72.84	-32.11	-60.24	-50.31
	adjusted-R^2	0.98	0.95	0.94	0.96	0.95	0.97	0.96
	RMSE	0.28	0.46	0.63	0.38	0.50	0.40	0.46
Weibull+tail	AICc	N.A.***	N.A.	-27.97	-68.39	-25.69	-56.68	-47.86
	adjusted-R^2	N.A.	N.A.	0.98	0.96	0.95	0.97	0.96
	RMSE	N.A.	N.A.	0.35	0.40	0.53	0.39	0.43
Biphasic model	AICc	-76.94	-62.46	-1.63	-21.05	-35.77	-19.62	-17.41
	adjusted-R^2	0.98	0.94	0.74	0.37	0.98	0.67	0.66
	RMSE	0.31	0.47	1.31	1.59	0.34	1.35	1.29

* SS: Saline Solution (0.85 %)
** AICc: Akaike´s Information Criterion
***N.A.: Not Adequate

Table 6. Regression parameters for log-linear model, log-linear with tail model, Weibull model, Weibull with tail model and biphasic model fitted to survival data of *Escherichia coli* O157:H7 on stainless steel inoculated with different substrates (chard, red cabbage, spinach, iceberg lettuce, parsley, romaine lettuce and saline solution).

Models	**Regression Parameters**	**Chard**	**Red cabbage**	**Spinach**	**Iceberg lettuce**	**Parsley**	**Romaine lettuce**	**Saline Solution**
Log-linear	K_{max}	0.06 (0.01)	0.05 (0.01)	0.39 (0.09)	0.06 (0.01)	0.31 (0.08)	0.10 (0.02)	0.11 (0.02)
	$Log_{10}(N0)$	5.84 (0.36)	5.72 (0.30)	6.26 (0.52)	5.52 (0.42)	5.69 (0.63)	5.76 (0.51)	5.35 (0.51)
Log-linear+tail	K_{max}	1.41 (0.35)	1.00 (0.26)	1.80 (0.28)	1.53 (0.36)	2.66 (0.58)	1.72 (0.33)	1.76 (0.35)
	$Log_{10}(N\_res)$	3.06 (0.25)	3.23 (0.20)	1.82 (0.44)	2.59 (0.26)	1.41 (0.44)	1.88 (0.30)	1.78 (0.31)
	$Log_{10}(N0)$	7.22 (0.35)	6.81 (0.28)	7.21 (0.29)	7.09 (0.36)	7.18 (0.42)	7.45 (0.37)	6.90 (0.36)
Weibull	delta	N.A.*	0.04 (0.04)	0.02 (0.03)	0.002 (0.004)	0.003 (0.005)	0.004 (0.008)	0.01 (0.01)
	p	N.A.	0.20 (0.02)	0.28 (0.04)	0.17 (0.03)	0.22 (0.04)	0.20 (0.03)	0.21 (0.04)
	$Log_{10}(N0)$	N.A.	7.80 (0.26)	8.49 (0.48)	8.53 (0.51)	8.81 (0.63)	8.88 (0.57)	8.22 (0.61)
Weibull+tail	Log_{10} (N_res)	N.A.	1.92 (1.22)	1.77 (0.16)	2.57 (0.21)	1.13 (0.26)	-1.39 (90.93)	1.75 (0.25)
	delta	N.A.	0.04 (0.05)	0.14 (0.06)	0.06 (0.10)	0.05 (0.04)	0.004 (0.009)	0.14 (0.18)
	p	N.A.	0.20 (0.03)	0.43 (0.05)	0.33 (0.11)	0.38 (0.07)	0.20 (0.04)	0.43 (0.13)
	$Log_{10}(N0)$	N.A.	7.79 (0.28)	8.21 (0.21)	8.35 (0.60)	8.50 (0.44)	8.88 (0.61)	7.91 (0.57)
Biphasic model	f	0.99 (0.00)	0.99 (0.00)	1.00 (0.00)	1.00 (0.00)	N.A.	1.00 (0.00)	1.00 (0.00)
	$K_{max}1$	3.32 (0.30)	1.41 (0.34)	3.37 (0.63)	0.32 (0.13)	N.A.	1.14 (0.51)	0.40 (0.20)
	$K_{max}2$	0.03 (0.00)	0.03 (0.00)	0.18 (0.04)	0.01 (0.03)	N.A.	0.05 (0.03)	0.04 (0.08)
	$Log_{10}(N0)$	7.65 (0.10)	6.92 (0.18)	7.57 (0.24)	8.13 (0.70)	N.A.	8.27 (0.59)	7.69 (0.77)

*N.A.: Not Adequate

Table 7. Regression parameters for log-linear model, log-linear with tail model, Weibull model, Weibull with tail model and biphasic model fitted to survival data of *Salmonella* on stainless steel inoculated with different substrates (chard, red cabbage, spinach, iceberg lettuce, parsley, romaine lettuce and saline solution).

Models	**Regression Parameters**	**Chard**	**Red cabbage**	**Spinach**	**Iceberg lettuce**	**Parsley**	**Romaine lettuce**	**Saline Solution**
Log-linear	K_{max}	0.08 (0.01)	0.07 (0.01)	0.29 (0.07)	0.06 (0.01)	0.28 (0.06)	0.08 (0.01)	0.10 (0.02)
	$Log_{10}(N0)$	5.94 (0.30)	6.00 (0.28)	5.71 (0.56)	5.40 (0.37)	5.74 (0.46)	5.53 (0.42)	5.19 (0.43)
Log-linear+tail	K_{max}	0.14 (0.03)	0.12 (0.03)	1.93 (0.27)	1.21 (0.37)	1.50 (0.32)	0.30 (0.06)	1.42 (0.24)
	$Log_{10}(N\_res)$	1.74 (0.40)	2.21 (0.45)	1.26 (0.35)	2.52 (0.28)	1.56 (0.46)	1.57 (0.32)	1.75 (0.24)
	$Log_{10}(N0)$	6.27 (0.29)	6.23 (0.31)	6.93 (0.28)	6.83 (0.44)	6.65 (0.37)	6.37 (0.31)	6.52 (0.27)
Weibull	delta	0.17 (0.13)	0.18 (0.25)	0.01 (0.02)	0.02 (0.03)	0.03 (0.04)	0.07 (0.07)	0.03 (0.04)
	p	0.27 (0.03)	0.26 (0.04)	0.25 (0.05)	0.21 (0.03)	0.27 (0.04)	0.25 (0.03)	0.23 (0.03)
	$Log_{10}(N0)$	7.75 (0.24)	7.69 (0.40)	8.26 (0.60)	7.94 (0.37)	7.88 (0.47)	7.91 (0.37)	7.53 (0.43)
Weibull+tail	$Log_{10}(N\_res)$	N.A.*	N.A.	1.23 (0.20)	1.20 (1.0)	0.76 (1.00)	1.02 (0.32)	1.16 (0.31)
	delta	N.A.	N.A.	0.16 (0.11)	0.03 (0.04)	0.04 (0.06)	0.12 (0.13)	0.06 (0.08)
	p	N.A.	N.A.	0.46 (0.08)	0.21 (0.03)	0.28 (0.06)	0.28 (0.04)	0.27 (0.05)
	$Log_{10}(N0)$	N.A.	N.A.	7.87 (0.32)	7.91 (0.39)	7.86 (0.50)	7.83 (0.37)	7.44 (0.41)
Biphasic model	f	1.00 (0.00)	0.99 (0.01)	1.00 (0.00)	1.00 (0.00)	0.99 (0.00)	1.00 (0.00)	1.00 (0.00)
	$K_{max}1$	2.18 (0.48)	1.17 (0.42)	1.22 (0.52)	0.25 (0.10)	5.33 (1.00)	0.27 (0.09)	0.52 (0.37)
	$K_{max}2$	0.05 (0.00)	0.05 (0.01)	0.03 (0.17)	0.02 (0.03)	0.17 (0.02)	0.03 (0.04)	0.01 (0.04)
	$Log_{10}(N0)$	7.23 (0.17)	6.97 (0.24)	7.32 (0.60)	7.73 (0.64)	7.35 (0.21)	7.55 (0.55)	6.93 (0.55)

*N.A.: Not Adequate

Conclusions

The present study evidenced that both pathogens, *E. coli* O157:H7 and *Salmonella* spp. were able to survive on stainless steel soiled with different vegetable juices from 1 to 8 days following a concave upward survival pattern and showing a tailing effect. The parsley and spinach juice substrates reduced survival capacity to only 1-2 days in comparison to the rest of substrates, particularly with respect to saline solution, which simulates cleaning conditions. This finding would suggest that these vegetable juices could contain certain antimicrobial substances affecting cell viability on surfaces. These substances could be polyphenolic compounds and others molecules belonging to the defence system of plants against infections. However, to obtain more precise data and conclusions, specific experiments should be carried out to determine such substances and their action mechanisms. Finally, the Weibull model and Weibull+tail models seemed to be the most suitable mathematical functions describing survival of both microorganisms in the different substrates. The use of these models allows estimating the available/viable cells for cross contamination through time if a contamination by one of the pathogens is produced at factory. Integrating these survival models into quantitative studies will lead to a better understanding of cross contamination, in addition to enabling the assessment of certain control measures such as sanitation regimes or disinfection processes on the final risk.

Acknowledgements

This work was partly financed by MICINN AGL2008-03298/ALI, the Excellence Projects AGR-01879 and P08-CTS-3260 (Junta de Andalucía), the EU project from the Seventh Framework Program KBBE 222738 Food, Agriculture and Fisheries, and Biotechnology and the Research Group AGR-170 HIBRO of the "Plan Andaluz de Investigación, Desarrollo e Innovación" (PAIDI), co-funded by the European Regional Development Fund (ERDF). International Campus of Excellence in the AgriFood Sector ceiA3. G.D. Posada-Izquierdo is holder of a predoctoral scholarship

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Chapter VI: "Modelling growth of *Escherichia coli* 0157:H7 in extract of different leafy vegetables"

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Published in: Predictive Modelling of Food Quality and Safety 2011 – Conference Proceedings, ISBN 1 900454 46 7

Abstract

Microbial risk derived from consumption of minimally processed vegetables is a serious concern for industry and governments. *Escherichia coli* O157:H7 is a food-borne pathogen which has been recently linked to several outbreaks associated with the consumptions of minimally processed vegetables. This pathogen can contaminate produces at harvest, and then, survive and/or grow during manufacturing, distribution, and storage, reaching the end consumer. The present work aims at studying and modelling the potential growth of *E. coli* O157:H7 in extract of different leafy vegetables at different storage temperatures.A cocktail including five *E. coli* O157:H7 strains resistant to nalidixic acid (NalR+) was built. Sterile extract from different leafy vegetables (iceberg lettuce, chard, spinach, parsley and romaine lettuce) was supplemented with nalidixic acid (50 μg/mL) and inoculated by the NalR+ pathogen cocktail (≈ 10^6 cfu/mL) in micro-plates (10x10 wells) and then incubated at different temperatures (4, 8, 10, 13, 16, and 20°C). The growth was monitored by absorbance measurement (8 replicates) by using Bioscreen C. Based on the observed absorbance data in the growth exponential phase, maximum growth rates and secondary models were estimated by using Excel Microsoft ®. Results indicated that the pathogen was able to grow in all assayed vegetable extracts. However, at 8°C, growth was only observed for parsley and chard. The fastest growth was obtained in chard extract (e.g. 0.26 h^{-1} at 20°C), followed by spinach (e.g. 0.12 h^{-1} at 20°C). The slowest growth was obtained in parsley extract (e.g., 0.012 h^{-1} at 20°C), although, in this extract, the microorganisms was able to grow at 8°C (0.001 h^{-1}). Finally, estimated maximum grow rates were used to derive a secondary model describing maximum growth rate as a function of temperature. The Ratkowsky´s model showed better convergence to observed data. The best fitting was obtained for spinach and chard extracts (R^2>0.85). Furthermore, the study provides evidence that compounds contained in vegetable tissues can result in a distinct growth niche producing different response in various types of vegetable.

Introduction

In recent years, consumer trends have shifted focus to healthier diets, increasing demand for natural products (or processed, that at least appear, such as salads RTE), especially leafy raw vegetables. This type of products can become contaminated by foodborne pathogens such as *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Gleeson & O`Beirne, 2005) at various stages of the food chain from "farm to table". However, no heat treatment or other inactivation method is applied which can guarantee a complete elimination of pathogenic microorganisms when presenting in produces. Hence, the incidence of illnesses transmitted by vegetables has been increasing as a result of these changes in consumption habits.

E. coli O157:H7 has been linked to outbreaks of various leafy vegetables such as lettuce, spinach, parsley, etc. (EFSA, 2009). Therefore, the aim of this study was to study and model the potential growth of *E. coli* O157: H7 in different leafy vegetables, which have not been studied extensively by scientific literature so far. In addition, the work looks for to give more information based on predictive microbiology and expanding the tools available that will enable us to assess the microbiological risks more effectively and to implement corrective action from the knowledge of the behavior of this pathogen.

Materials and methods

Growth medium

Sterile vegetable extracts were used to simulate growth of the pathogen in leafy vegetable matrices. To obtain the extract, first different vegetables (chard, spinach, parsley, iceberg and romaine lettuce) were homogenized in distilled water with a proportion 1:3 (vegetable/water) by using Stomacher. Then, generated extracts were sterilized by filtration through a step-by-0.22 micron membrane (Millipore filter unit-Express Plus PES). Extracts of each vegetable were plated to confirm sterility.

Bacterial strains and inoculum preparation

A cocktail of five strains of *Escherichia coli* O157: H7 (CECT 4076, 4267, 4782, 4783 and 5947) was used in this study. Cocktail trains were previously made resistant to at 50 μg/mL of Nalidixic acid (NaL) (Merck, Darmstadt, Germany) (Allende et al, 2008). Prior to growth experiments, cultures were grown in Tryptic Soy Broth (TSB) at 37 ° C for 18-20 h in three incubation loops, and then mixed at equal volumes of cell suspensions to give approximately equal populations of each culture. Then, the cocktail was washed three times by centrifugation (4100 g) and suspended in phosphate buffer (PBS) obtaining an inoculum level of 10^8 cfu/mL, approximately. Counts were obtained by growth on McConkey-Sorbitol, MCS agar and on Tryptone Bile X-Glucuronide Medium, TBX agar (Oxoid, UK) supplemented with Nal (50 μg/mL).

Inoculation procedure and assessment of growth

The Bioscreen C (Labsystems, Finland) was used to monitor bacterial growth based on absorbance measures at 420-580 nm. Cocktail of *E. coli* O157: H7 previously washed and resuspended in PBS was diluted 1:100 in vegetable extracts supplemented with NaL (50 μg/mL) obtaining a concentration of 10^6 cfu/mL, approximately. Micro-plates (10x10 wells) belonging to Bioscreen C were utilized to perform the growth experiment. Each well was filled with 300μl of inoculated vegetable extracts with a total of eight replicates and two blanks per extract. The plates were incubated at different temperatures (4, 8, 10, 13, 16 and 20°C) during a period of 21 days. At high temperatures (13, 16 and 20 °C), growth was monitored continuously by Bioscreen C, while at lower temperatures, absorbance measurements were made at specific time points during experiments (8 and 10°C). Growth observed in wells was confirmed by plating an extract aliquot on MCS agar supplemented with NaL.

Growth modelling

Maximum growth rates were estimated based on the observed absorbance data (log) in the growth exponential phase by using Excel Microsoft ®. Secondary models were

fitted to maximum rates using the DMFit program (Excel Add-In) (Baranyi & Roberts, 1994).

Results and Discussion

Growth was not detected in romaine and iceberg vegetable extracts at all temperatures. However, parsley, spinach and chard presented significant increase of absorbance for all temperatures excepting for 4ºC at which no growth was detected in all extracts. Chard extract did not support *E. coli* O157: H7 growth at 8ºC. Likewise for assays at 10ºC in the same extract, only few replicates presented a significant increase of absorbance, within the linearity range (>0.074); although growth data was not enough to appropriately estimate the maximum growth rate. *E. coli* O157:H7 in spinach at 8ºC showed a digenetic behavior, in which 8 out of 4 wells presented a significant growth. To model growth in chard extract, temperatures 8 and 10ºC were discarded, while for spinach only positive replicates at 8º C were considered.

Overall, the pathogen presented different growth patterns in the different vegetable extracts. The fastest growth was obtained in chard extract (0.26 h^{-1} at 20°C), followed by spinach (0.12 h^{-1} at 20°C). In turn, the slowest growth was observed in parsley extract (0.012 h^{-1} at 20°C); although in this extract the microorganism was able to grow at 8°C (0.001 h^{-1}). There are few studies in scientific literature dealing with these food matrices and E. coli O157:H7 growth. Growth rates observed, in our study, at refrigeration temperatures (8-13 °C) were low when compared to other studies (Valero et al. 2010). For instance, Koseki & Isobe (2005) reported a growth rate of 0.03 (h^{-1}) for *E. coli* O157:H7 in lettuce at 10ºC, while in our study, at this temperature, the growth rate oscillated between 0.002 and 0.004 h^{-1}. Similarly, Rowaida and Josepth (2010) found higher growth of E. coli O157:H7 in damaged spinach storage for 3 days at 8 and 12ºC with increases of 1.18 and 2.08 log cfu/g, respectively. By contrary, at high temperatures (20º C), growth rates were quite similar to those reported by other studies. For instance, the study by Koseki & Isobe (2005) showed a maximum growth rate of 0.26 h^{-1} in lettuce leaves stored at 20ºC which was equal to the value obtained in our study for chard extract at the same temperature (i.e. 0.26 h^{-1}).

Surprisingly, romaine and iceberg lettuce did not present any growth during 21 days. This result is not in concordance with that reported by most studies which demonstrate a significant growth in the temperature range 10-25º C (Koseki & Isobe, 2005). However, these studies were mostly performed on inoculated vegetable surfaces, and not in aqueous extracts of vegetable, which contain a complex and concentrated mixture of substances released from vegetable tissues (peptides, phenols, fiber, enzymes, etc.). Regarding this, the study Rowaida and Josepth (2010) found that *E. coli* 057:H7 was not able to grow on damaged leaves of romaine lettuce at 8 and 12ºC, but growth was observed at 15ºC. This study hypothesized that the inhibition at low temperatures could be caused by oxidation reactions associated with tissue damaged. In fact, it is known that some vegetable species can present substances with antimicrobial activity (Hashem & Saleh, 1999). Besides that, it cannot be discarded that both sample of romaine and iceberg lettuce were contaminated with pesticides with antimicrobial activity.

Finally, estimated maximum grow rates were used to derive a secondary model

describing maximum growth rate as a function of temperature. The Ratkowsky´s model (Ratkowsky et al, 1982) showed better convergence to observed data in all extracts. For spinach and parsley, the best fitting was obtained when square root was applied to maximum growth rate, while for chard extract the best fitting was attained when no mathematical transformation was used. Regression parameters (b and T_{min}) and Standard Error of the Ratkowsky´s model for the E. coli O157:H7 growths in the three extracts are showed in Table 1.

Table 1. Estimated regression parameters of the Ratkowsky´s model based on *E. coli* O157:H7 growth in different vegetable extracts.

Vegetable Extract	Temperature range (ºC)	*b*	T_{min}	SE*
Chard	13-20	5.00×10^{-03}	13.0	3.4×10^{-02}
Parsley	8-20	4.64×10^{-05}	2.9	5.6×10^{-03}
Spinach	8-20	9.97×10^{-04}	8.7	4.8×10^{-02}

SE: Standard Error

Conclusions

Results indicated that *E. coli* O157:H7 was able to grow in different aqueous extracts of vegetables in a broad range of temperature, although growth pattern varied depending on the type of extract. Use of vegetable extracts can help to better simulate conditions given in vegetable tissues where bacteria can reside (internalization or injury), survive and growth thereby allowing the pathogen transmission through the food chain. Further, results suggest that unknown compounds present in vegetable extracts could exert an inhibition effect on *E. coli* O157:H7 growth at low temperatures. However, further study will be needed to confirm the existence of potential antimicrobial substance in these types of vegetable.

Acknowledgements

The research has received funding from (project AGL2008-03298), the AGR-170Research Group, HIBRO and the MCINN with a predoctoral scholarship for Guiomar Posada-Izquierdo.

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Conclusions/Conclusiones

1. According to an *in-silico* cross-contamination simulation, the pathogen *Escherichia coli* O157:H7 is capable to transmit along processing line and remain and/or cross-contaminate final fresh-cut leafy vegetables even when unprocessed vegetable is contaminated at very low levels (0.01 log cfu/g) even though in this case, contamination occurs sporadically. (*Chapter I*)

2. The probabilistic cross-contamination model also showed that the pathogen *Escherichia coli* O157:H7 was able to be present in the final fresh-cut leafy vegetables for all simulated interventions scenarios although irradiation (0.5 KGy) was a more effective decontamination step in reducing prevalence than chlorination or pathogen testing under the same simulated conditions. These results also suggest that different preventive measurements (chlorination, water recycling, etc.) should jointly applied in order to mitigate the risk by *Escherichia coli* O157:H7 in fresh-cut leafy vegetables. (*Chapter I*)

3. The study of an innovative disinfection method based on electrolyzed water using boron-doped diamond (BDD) determined that the method was effective in reducing *Escherichia coli* O157:H7 in tap water with different levels of organic matter 60, 300, 600 and 800 mg/L of COD (Chemical Oxygen Demand). A maximum log-reduction of 5 log units was observed in all cases, which was attained faster at lower COD values as well as when tap water was supplemented with NaCl. (*Chapter II*)

4. Inactivation of *Escherichia coli* O157:H7 by electrolyzed water with BDD electrodes exhibited a non-linear kinetic, presenting three phases: shoulder + exponential decay + tail. This inactivation pattern can be well described by the biphasic+shoulder model and the Weibull model. (*Chapter II*)

5. The treatment of disinfection affected the capacity of growth of *Escherichia coli* O157:H7 on fresh-cut lettuce packaged under modified atmosphere. The growth observed after treatment with electrolyzed water was lower than that obtained with chlorinated water (150 ppm). (*Chapters III and IV*)

6. The pathogen *Escherichia coli* O157:H7 was able to grow at a temperature as low as 8 ºC on fresh-cut lettuces packaged under modified atmosphere and previously treated both with chlorinated water and electrolyzed water. Nonetheless, at this refrigeration temperature the pathogen evidenced a more variable behavior with some replicates showing no growth. (*Chapters III and IV*)

7. A Ratkowsky-type model has been developed to accurately describe growth of *Escherichia coli* O157:H7 as a function of temperature in extract of different vegetables and on fresh-cut lettuce packaged under modified atmosphere taking into consideration the effect of disinfection treatments with electrolyzed water and chlorinated water. (*Chapters III, IV and VI*)

8. The capacity of survival of *Escherichia coli* O157:H7 and *Salmonella* spp. on stainless steel surfaces soiled with different vegetable substrates depended on the type of vegetable. Both microorganisms were able to survive up to until 192

and 168 h, respectively on surfaces soiled with chard, red cabbage, iceberg lettuce and romaine lettuce. (*Chapter V*)

9. Stainless steel surfaces soiled with parsley and spinach vegetable juices resulted in a lower survival ability of *Escherichia coli* O157:H7 and *Salmonella* spp. than that observed in surfaces simulating cleaning conditions (i.e. soiled with saline solution), reaching maximum survival times of less than 48 hours. (*Chapter V*)

10. The Weibull model could well describe survival kinetics of *Escherichia coli* O157:H7 and *Salmonella* spp. on stainless steel surfaces soiled with different vegetable juices. The application of survival models is crucial to better represent the impact of cross contamination scenarios in studies of risk assessment. (*Chapter V*)

11. The information derived from the present thesis, describing the impact of different food processes on pathogenic enteric bacteria is suitable to support, on a quantitive base, decision-making processes in risk management systems in the IV gamma vegetable Industry. (*All Chapters V*)

1. Según la simulación *in-silico* del modelo probabilístico de contaminación cruzada de *Escherichia coli* O157:H7 en vegetales IV Gama, el patógeno fue capaz de transferirse a lo largo de la línea de procesado y alcanzar el producto final, incluso cuando los vegetales sin procesar fueron simulados con niveles bajos de contaminación (0.01 log ufc/g), si bien en este caso, la contaminación ocurrió de manera esporádica. (*Capítulo I*)

2. El modelo probabilístico de contaminación cruzada también demostró que *Escherichia coli* O157: H7 fue capaz de contaminar el producto final para todas las estrategias de mitigación o intervención simuladas. No obstante la irradiación (0,5 KGy) fue la medida de control más eficaz en comparación con el tratamiento con agua clorada y el muestreo e investigación del patógeno en el producto final. Estos resultados sugieren que la mayor eficacia en la reducción del riesgo del patógeno se obtendría mediante la aplicación conjunta de diferentes tecnologías y medidas preventivas. (*Capítulo I*)

3. El estudio de un método de desinfección innovador basado en agua electrolizada neutra usando electrodo de diamante-Boro (BDD) determinó que el método fue eficaz en la reducción de *Escherichia coli* O157: H7 en el agua de lavado con diferentes niveles de materia orgánica 60, 300, 600 y 800 mg/L de DQO (Demanda Química de Oxígeno). Una reducción máxima de 5 unidades logarítmicas fue observada en todos los casos, alcanzándose más rápido para los valores más bajos de DQO y para el agua del grifo suplementada con NaCl. (*Capítulo II*)

4. La inactivación de *Escherichia coli* O157: H7 con agua electrolizada neutra, generada con un electrodos BDD exhibió una cinética no-lineal, presentando tres fases: hombro, declive exponencial y cola. Este patrón de inactivación puedo ser descrito adecuadamente por el modelo bifásico con hombro y el modelo de Weibull. (*Capítulo II*)

5. El tratamiento de desinfección afectó a la capacidad de crecimiento de *Escherichia coli* O157: H7 en la lechuga cortada y envasada en atmósfera modificada. El crecimiento del patógeno después de un tratamiento con agua electrolizada fue significativamente menor que el obtenido con agua clorada (150 ppm). (*Capítulos III y IV*)

6. *Escherichia coli* O157: H7 fue capaz de crecer a una temperatura tan baja como 8 ºC sobre lechuga IV Gama envasadas en atmósfera modificada, tratada previamente con agua clorada y agua electrolizada. Sin embargo, a esta temperatura de refrigeración el patógeno evidenció un comportamiento más variable y en algunas repeticiones no mostró crecimiento. (*Capítulos III y IV*)

7. Un modelo de tipo Ratkowsky ha sido desarrollado con buenos resultados para describir, en función de la temperatura, el crecimiento de *Escherichia coli* O157:H7 en extracto de diferentes vegetales y en lechuga IV Gama envasada en atmósfera modificada, considerando el efecto de los

tratamientos de desinfección con agua electrolizada y clorada. (*Capítulos III, IV y VI*)

8. La capacidad de supervivencia de *Escherichia coli* O157:H7 y *Salmonella* spp. en superficies de acero inoxidable impregnadas con diferentes extractos vegetales depende del tipo de vegetal. Ambos microorganismos pudieron sobrevivir hasta 192 y 168 h, respectivamente sobre superficies impregnadas con acelga, col lombarda, lechuga iceberg y lechuga romana.(*Capítulo V*)

9. Las superficies de acero inoxidable impregnadas con extracto de perejil y espinacas resultaron en una menor capacidad de supervivencia de *Escherichia coli* O157: H7 y *Salmonella* spp. en comparación con las observadas en las superficies que simulan condiciones de limpieza (solución salina), alcanzando un tiempo máximo de supervivencia de menos de 48 h. (*Capítulo V*)

10. El modelo Weibull pudo describir con mayor exactitud la cinética de supervivencia de *Escherichia coli* O157: H7 y *Salmonella* spp. en superficies de acero inoxidable impregnadas con extractos de diferentes vegetales. La aplicación de modelos matemáticos de supervivencia es crucial para obtener una representación más exacta, en los estudios de evaluación de riesgos, del impacto de los diferentes escenarios de contaminación cruzada. (*Capítulo V*)

11. La información derivada de la presente tesis, describiendo el impacto de los diferentes procesos alimentarios sobre las bacterias patógenas entéricas, es muy adecuada para fundamentar con base cuantitativa la toma de decisiones en la gestión del riesgo en la industria de vegetales IV Gama. (*Todos los Capítulos*)

Agradecimientos

A lo largo de mi vida he tenido la suerte de toparme por casualidad o por el destino con personas únicas e inolvidables sea cual sea mi dirección postal, la aventura de venir a España, a este maravilloso país nunca había tenido mas sentido que hasta ahora, por fin aquí esta mi tesis Doctoral, pero el camino andado hasta llegar aquí nunca hubiera sido posible sin la ayuda o "el compartir" con personas, amigos, compañeros y jefes aquienes les quiero dedicar unas pequeñísimas líneas porque podría escribir otra tesis solo para los agradecimientos a todos ustedes. En resumen para no liarme hablando simplemente es decirles GRACIASssss!!! de corazón muchas gracias y los tendre siempre presente en mis recuerdos.

Curriculum Vitae

Ingeniera Agronoma: **Guiomar Denisse Posada Izquierdo**

SCIENTIFIC CONTRIBUTIONS IN CONFERENCES

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