Aplicación de la Microbiología Predictiva en el estudio del impacto de la calidad del agua de lavado y desinfección sobre la seguridad microbiológica de vegetales listos para consumo

Application of Predictive Microbiology in the study of the impact of the quality of washing and disinfection water on the microbiological safety of ready-to-eat vegetables

Tesis doctoral presentada por

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Tesis doctoral en régimen de cotutela entre la Universidad de Córdoba, España y la Facultad de Ciencias Agropecuarias de la Universidad Nacional de Córdoba, Argentina.

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Esta Investigación se desarrolló en cotutela entre la Universidad Nacional de Córdoba, España y la Universidad Nacional de Córdoba, Argentina. Se firmó un convenio específico de cotutela el día 28 de enero de 2018. El trabajo experimental se llevó a cabo en el Departamento De Bromatología y Tecnología de los Alimentos, de la Facultad De Veterinaria. Universidad de Córdoba, España.

TÍTULO DE LA TESIS: Aplicación de la microbiología predictiva en el estudio del impacto de la calidad del agua de lavado y desinfección sobre la seguridad microbiológica de vegetales listos para consumo

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

La presente tesis se enmarca dentro de un convenio de co-tutela entre la Universidad de Córdoba (UCO) de España y la Universidad Nacional de Córdoba (UNC) de Argentina. Además, la Asociación Universitaria Iberoamericana de Posgrado (AUIP), mediante el programa de Formación Doctoral en Agroalimentación y Ciencia Veterinarias, financió tres estancias de la doctoranda la Universidad de Córdoba, España.

La doctoranda Dña. Sofia Griselda Cuggino, ha realizado satisfactoriamente el trabajo de investigación presentado en esta memoria de tesis habiendo alcanzado todos los objetivos planteados en el proyecto de investigación. Es importante resaltar que desarrollar un trabajo de investigación en dos universidades de reconocido prestigio, tiene, por un lado, una riqueza académica muy importante y a su vez, el desafió de organizar los tiempos de cada estancia y poder cumplir con los objetivos planteados.

La doctoranda realizó tres estancias en el Departamento de Bromatología y Tecnología de los Alimentos de la UCO en los laboratorios del grupo HIBRO, donde llevó a cabo el desarrollo experimental, puesta a punto de métodos, obtención de resultados, y análisis preliminares de los datos, para cumplimentar los objetivos propuestos. Además, en la Facultad de Ciencias Agropecuarias de la UNC realizó las tareas de actualización bibliográfica, análisis de datos, discusión de resultados, redacción y publicación de artículos, así como también la recogida de información gracias a las visitas de diversas empresas del sector.

El trabajo desarrollado en esta tesis logra traer respuestas a las realidades y problemáticas de las industrias de vegetales listos para consumo españolas y argentinas, que por momentos parecen muy diferentes pero que en muchos “puntos críticos” se asemejan. Para dar respuestas y soluciones a algunas de las problemáticas comunes se utilizó la herramienta de la microbiología predictiva. Esta herramienta se aplica, principalmente, a los procesos de higienización, y a su impacto en la calidad y seguridad de los productos vegetales de hoja listos para el consumo. Asimismo, se presta atención al uso de alternativas más seguras al hipoclorito, como desinfectante, y a su eficacia en la reducción y eliminación de patógenos alimentarios.

La tesis cuenta con un capítulo de revisión bibliográfica (Capítulo 1), donde se describe los aspectos esenciales del sector de vegetales listos para consumo en España y en Argentina, como un producto con un reciente crecimiento en este último caso. Además, se detallan los conceptos del Sistemas de Gestión de Calidad y Seguridad en la producción de vegetales, los riegos asociados a su producción, las herramientas y normativas vigentes para disminuir la contaminación y producir alimentos inocuos. Y finalmente se introduce la microbiología predictiva como herramienta que permite, entre otras cosas, predecir y anticiparse a riegos microbiológicos durante la elaboración de vegetales listos para consumo. Parte de este capítulo fue publicado con el título "Quality and Safety Management Systems in the Production of Vegetables" en el libro Quantitative Methods for Food Safety and Quality in the Vegetable Industry, de la prestigiosa editorial Springer en el 2018.

Partiendo del estado de situación descrito en el capítulo anterior, se realizó un estudio prospectivo para conocer las diferentes tipologías y tecnologías de procesos de lavado aplicados en la industria alimentaria de vegetales listos para consumo, identificando factores de riesgo y parámetros de proceso. Asimismo, se relacionaron los peligros de mayor incidencia y relevancia en este tipo de productos. Para abordarlo, la doctoranda realizó un análisis bibliográfico exhaustivo, y a su vez, recorrió, evaluó y caracterizó el proceso de elaboración de vegetales de hoja listos para consumo en una empresa en Argentina (Capítulo 3). Parte de los resultados se publicaron en una revista de difusión Socio-tecnológica, y en comunicaciones en eventos científicos (congresos y jornadas).

Una vez caracterizado el proceso de elaboración de los vegetales listos para consumo, el siguiente objetivo fue desarrollar y aplicar modelos experimentales y matemáticos, basados en la caracterización realizada, para estudiar y evaluar los procesos de lavado y desinfección y los parámetros aplicados en la elaboración de vegetales listo para el consumo. En este trabajo se considera el uso del desinfectante más usado, el hipoclorito, y un alternativa más sostenible y segura basada en el uso de compuestos naturales, como son los tiocianatos (Capítulo 4). El desarrollo de este capítulo dio lugar a una publicación titulada "Modelling the combined effect of chlorine, benzyl isothiocyanate, exposure time and cut size on the reduction of *Salmonella* in fresh-cut lettuce during washing process", en la revista Food Microbiology en el 2021.

La doctoranda aplicó modelos predictivos para el estudio del efecto de la lavado y desinfección sobre la supervivencia y posterior crecimiento del *Salmonella* en vegetales de hoja (Capítulo 5). En este trabajo se pone de relieve el efecto significativo que ejerce el tipo de desinfectante sobre el comportamiento del patógeno durante almacenamiento. Los resultados obtenidos se han presentado en el trabajo de título: "Effects of chlorine and peroxyacetic acid washing treatments on growth kinetics of Salmonella in fresh-cut lettuce under commercial storage conditions", que se ha enviado para su publicación a la revista Food Research International en el 2022.

Durante el año 2020, la situación global vivida por la alerta sanitaria debido a la pandemia del COVID-19, ocasionó una restructuración del desarrollo del último objetivo de la tesis. El objetivo planteaba aplicar los modelos predictivos recopilados y desarrollados, con datos obtenidos de situaciones reales de producción industrial de vegetales listos para consumo. Para obtener los datos reales, era necesario realizar visitas a las industrias, que se vieron afectados por el aislamiento social preventivo. Como solución se realizó contactos vía mail con las industrias y se desarrolló una encuesta utilizando herramientas virtuales. Finalmente, con los datos recopilados, se combinaron con los modelos de microbiología predictiva desarrollados y se evaluaron los efectos de las etapas y parámetros reales aplicados por las industrias sobre una posible contaminación con *Salmonella* (Capítulo 6).

Sofía Cuggino ha demostrado una excelente formación teórica y práctica, conociendo los fundamentos metodológicos y los procedimientos de microbiología de los alimentos necesarios para el desarrollo de proyectos en esta área. Ha contribuido sustancialmente al desarrollo científico de los grupos de trabajo, tanto de España como de Argentina. Durante todo el periodo de ejecución de la tesis, Sofia Cuggino ha tenido una actitud muy positiva, demostrando un gran interés por aprender y formarse en distintos aspectos que integran parte fundamental de esta etapa académica. Son destacables su actitud proactiva, su capacidad de trabajo, y su excelente integración a la dinámica de trabajo de los grupos de investigación en los que se desarrolló la tesis. Su gran generosidad ha sido una seña característica de la doctoranda.

Por lo mencionado, la conclusión de esta tesis doctoral deja las puertas abiertas tanto para el perfeccionamiento postdoctoral de Sofía Cuggino en los grupos de España y Argentina, como así también para futuras colaboraciones que permitan consolidar el vínculo entre los laboratorios e integrantes de ambos países, iniciado con motivo de la realización de esta tesis doctoral en co-tutela.

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

Córdoba, 18 de noviembre del 2022

Firma del/de los director/es

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"Si pudiera hablar todos los *idiomas del mundo*,
pero no **amara** a los demás,
yo sólo sería un metal que resuena
o un platillo que hace ruido"
(Apóstol Pablo Carta a los/as Corintios)

"Solo sirven las conquistas científicas sobre la salud si estas son accesibles al pueblo" (Ramón Carrillo~1946)

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Listado de abreviaturas y símbolos

AFHORLA: Asociación de Frutas y Hortalizas de IV Gama
AIB: The American Institute of Baking
ANMAT: Administración Nacional de Medicamentos, Alimentos y Tecnología Médica
ANVISA: Agencia Nacional de Vigilancia Sanitaria
AUIP: Asociación Universitaria Iberoamericana de Posgrado
BHI: Brain Heart Infusion
BITC: benzyl-isothiocyanate
BPM: Buenas Prácticas de Manufactura
BRC: British Retail Consortium
CAA: Código Alimentario Argentino
CCD: Central Composite Design
CCP: Critical Control Points
CFU: Colony Forming Unit
CO_2: Dióxido de carbono
CONAL: Comisión Nacional de Alimentos
DBP: Disinfection byproducts
EC: Commission Regulation
EFSA: European Food Safety Authority
EFSA: European Food Safety Authority
EFSA: European Food Safety Authority
FAO: Food and Agriculture Organization
FBD: Food-Borne Diseases
FCA: Facultad de Ciencias Agropecuarias
FCC: Free Chlorine Concentration
FDA: Food and Drug Administration
FoNAO: Foods of Non-Animal Origin
FSIS: Food Safety and Inspection Service
FSO: Food Safety Objective
GAP: Good Agricultural Practice
GFP: Green Fluorescent Protein
GFSI: Global Food Safety Initiative
GHP: Good Hygienic Practices
GMP: Good Manufacturing Practices
HACCP: Hazard Analysis and Critical Control Points
HCl: Chlorhydric acid
HOCl: Hypochlorous acid
Horeca: Hoteles, Restaurantes Y Servicio De Catering
IPM: Integrated Pest Management
ISO: International Organization for Standardization
ITCs: Isothiocyanates
MAP: Mmodified Atmosphere Packaging
MEA: Microbiological Exposure Assessment

MPD: Maximum Population Density
MPN: Most Probable Number
MRL: Maximum Residue Limits
MSR: Modelos De Superficie De Respuesta
NA: Not Apply
NaOCl: Sodium hypochlorite
NMP: Número Más Probable
NR: Not Reported
OVQ: Overall Visual Quality
PAA: Peroxyacetic acid
PAL: Phenylalanine Ammonia Lyase
PC: Performance Criteria
PCA: Plate Count Agar
PDO: Protected Designation of Origin
PGI: Protected Geographical Indication
PO: Performance Objectives
POD: Peroxidase
PPO: Polyphenol Oxidase
QM: Quality Management System
QMRA: Quantitative Microbial Risk Assessment
RA: Risk Assessment
RASFF: Sistema de Alerta Rápida para Alimentos y Piensos
RH: Relative Humidity
RSM: Response Surface Methodology
RTE: Ready-To-Eat
SAG: Servicio Agrícola y Ganadero
SEM: Scanning Electron Microscopy
SENASA: Servicio Nacional de Sanidad y Calidad Agroalimentaria
SH: Sodium hypochlorite
SPS: Sanitary and Phytosanitary
SSOP: Sanitation Standard Operating Programs
SWW: Simulated Wash Water
TBT: Technical Barriers to Trade
TDS: Total Dissolved Solids
THMs: Trihalomethanes
TOC: Total Organic Carbon
TSG: Traditional Specialties Guaranteed
UCO: Universidad de Córdoba
UFC: Unidades Formadoras de Colonias
UNC: Universidad Nacional de Córdoba
USDA: United States Department of Agriculture
VMP: Vegetales Mínimamente Procesados
WHO: World Health Organization
WTO: World Trade Organization

Resumen

En España, la industria dedicada al procesamiento, empaque y distribución de vegetales listos para consumo tiene una trayectoria de muchos años, con un constante crecimiento en su producción, como consecuencia de que el consumidor de vegetales fue incorporando en su dieta este tipo de productos. Cabe destacar que la producción de estos alimentos se ve centralizada en dos o tres grandes industrias que abastecen a todo el territorio, y el consumidor encuentra estos alimentos exhibidos en las góndolas de muchas cadenas de supermercados y en mercados de cercanía.

En Argentina la situación de las industrias dedicadas al procesamiento de estos productos es distinta a las empresas de España. Por un lado, la producción de vegetales listos para consumo es relativamente nueva. Las empresas que se dedican al procesamiento, envasado y distribución, por lo general lo hacen como agregado de valor de su propia producción primaria de vegetales y la tecnología aplicada no está totalmente automatizada. Además, el consumidor de vegetales aún no ha incorporado este tipo de producto en su dieta. Esto último puede deberse a su escasa accesibilidad y disponibilidad en el mercado, centralizado principalmente en las grandes ciudades.

El trabajo desarrollado en esta tesis intenta visualizar y traer respuestas a las realidades y problemáticas de las industrias de vegetales listos para consumo españolas y argentinas, que por momentos parecen muy diferentes pero que en muchos "puntos críticos" se asemejan. En ambos países, la calidad del agua utilizada en los procesos de desinfección y/o lavado, y los procesos en sí mismos, se tornan cruciales en el control de la contaminación microbiológica de estos productos, y particularmente, en la reducción de patógenos alimentarios.

Sobre esta base, como objetivo general, definido en el Capítulo 2, se propuso desarrollar modelos predictivos para el estudio de los procesos de lavado y desinfección de vegetales listos para consumo, identificando combinaciones de parámetros más efectivas sobre la calidad y seguridad del producto final.

Para alcanzar el objetivo, en primera instancia se realizó una revisión bibliográfica (Capítulo 1), en la que se profundizó sobre las características de la cadena de producción de vegetales listos para consumo en Argentina y en España. Además, se incluyó información sobre los principales aspectos de la calidad microbiológica de estos productos y los conceptos fundamentales de los Sistemas de Gestión de Calidad y Seguridad en la producción de

vegetales. También se definieron las normativas vigentes para disminuir la contaminación microbiana y los riesgos asociados a la producción y transformación de vegetales. Finalmente, se realizó un análisis de la microbiología predictiva como herramienta para garantizar la inocuidad de estos productos, proporcionando una descripción resumida de los procesos de generación de modelos matemáticos y su aplicación como herramienta para predecir y anticiparse a un riesgo microbiológico durante la elaboración de vegetales listos para consumo. Parte de este capítulo ha sido publicado con el título "*Quality and Safety Management Systems in the Production of Vegetables*", en el libro Quantitative Methods for Food Safety and Quality in the Vegetable Industry, Springer.

Con el objetivo de conocer las diferentes tipologías y tecnologías del proceso aplicados en la industria de vegetales listos para consumo, e identificar los factores de riesgo y parámetros de mayor incidencia y relevancia en la calidad microbiológica de esos productos, se realizó una caracterización y evaluación del cumplimiento de las Buenas Prácticas de Manufactura en una empresa de vegetales listos para consumo de Argentina (Capítulo 3). Además, se determinó la calidad microbiológica de los puntos críticos y de los productos elaborados y almacenados. Como resultado se publicó un artículo en la revista NEXO Agropecuario con el título "*Optimización del proceso de elaboración de rúcula lista para consumo*".

En base a los resultados obtenidos de la caracterización del proceso de elaboración de los vegetales listos para consumo, alcanzados en los capítulos 1 y 3, se investigó sobre los efectos de los procesos de desinfección en la inactivación de *Salmonella* enterica sv Thompson en lechuga iceberg. Pare ello, en una primera instanacia, se estudió la capacidad desinfectante del hipoclorito de sodio (0-200 ppm), y de una alternativa más sostenible y segura basada en el uso de compuestos naturales, como es el isotiocianato de bencilo (0-80 ppm).

Los resultados obtenidos, se utilizarón para construir un modelo predictivo, y de caracter polinomial, con la finalidad de evaluar y cuantificar el efecto de la combinación de los desinfectantes y los parámetros del proceso (tamaño de corte, tiempo de contacto y temperatura del agua), en la reducción de *Salmonella*. Además, el modelo desarrollado se aplicó en un esquema cuantitativo de Evaluación de Exposición Microbiológica para determinar el impacto del tratamiento de desinfección en los niveles de exposición a *Salmonella* en lechuga, cuando un lote contaminado ingresa a la línea de procesamiento. Para ello se construyó un modelo probabilístico en MICROHIBRO (Capítulo 4). Como resultado

se obtuvo una publicación titulada "*Modelling the combined effect of chlorine, benzyl isothiocyanate, exposure time and cut size on the reduction of Salmonella in fresh-cut lettuce during washing process*", en la revista internacional Food Microbiology.

En un segundo trabajo se evaluó la reducción y/o eliminación de *Salmonella* enterica sv Thompson en lechuga cortada, tratada con agua de lavado simulada, hipoclorito de sodio (cloro libre 25 mg/L) y ácido peroxiacético (80 mg/L). Además de analizar la eficacia de los desinfectantes para inactivar al microorganismo, se evaluaron sus efectos sobre la calidad sensorial y sobre el posterior crecimiento bacteriano durante el almacenamiento a 9, 13 y 18 ºC, simulando condiciones de refrigeración con abuso de temperatura representando una situación de riesgo para este patógeno.

En base a los datos experimentales obtenidos en la fase de almacenamiento, y utilizando el modelo primario de crecimiento de Baranyi y Roberts, se describió la cinética de *Salmonella* en las temperaturas de almacenamiento estudiadas. Posteriormente, se utilizaron modelos secundarios basados en el modelo de Bělehrádek para representar la relación entre las tasas de crecimiento y la temperatura. Fue destacable como resultado que la exposición a desinfectantes determinó el tipo de cinética de crecimiento de *Salmonella* durante el almacenamiento (Capítulo 5). A partir de los datos generados se redactó un trabajo con el título: "*Effects of chlorine and peroxyacetic acid washing treatments on growth kinetics of Salmonella in fresh-cut lettuce under commercial storage conditions*", aceptado para su publicación a la revista Food Research International.

Finalmente, con el objetivo de aplicar los modelos de microbiología predictiva desarrollados en capítulos previos, se realizó un trabajo donde, en un primer lugar, se recopiló información sobre las condiciones y prácticas de procesamiento empleadas en siete industrias de vegetales listos para consumo de Argentina. Estos datos se utilizarón con modelos de microbiología predictiva para estimar los niveles de *Salmonella* después del proceso de desinfección, y durante el almacenamiento y la distribución de los productos. Este estudio permitió evaluar el riesgo de contaminación asociado a las operaciones, parámetros y condiciones de los procesos utilizados en la industria de vegetales (Capítulo 6). Los resultados de este trabajo se publicarán en una revista científica con la finalidad de dar a conocer los procesos reales aplicados, y a su vez se compartirán con las empresas participantes del estudio, para acercarles el conocimiento desarrollado en base a sus realidades.

En un capítulo final se describen todas las conclusiones obtenidas del desarrollo de esta tesis doctoral (Capítulo 7).

Los resultados de este trabajo de investigación se publicaron y dieron a conocer a nivel nacional e internacional, demostrando la calidad de la investigación y el trabajo conjunto de ambas universidades. Asímismo, se realizaron presentaciones escritas y orales en eventos científicos (congresos y jornadas). Además, los resultados obtenidos constituyen una fuente de información relevante para la comunidad científica, el sector industrial, y las autoridades sanitarias en materia de calidad y seguridad alimentaria de ambos países.

Palabras claves: vegetales mínimamente procesados, microbiología predictiva, cinética de crecimiento, *Salmonella*, lechuga, evaluación del riesgo microbiano, desinfectante, industria.

Diagrama que resume el contenido de la Tesis:

1era PARTE: Introducción bibliográfica (capítulo 1)

Vegetales listos para consumo/IV Gama. Consumo y producción en Argentina y en España.
Sistemas de Gestión de Calidad y Seguridad en la producción de hortalizas
Microbiología predictiva. Tipos de modelos. Modelos primarios, secundarios y terciarios. Etapas para el desarrollo de los modelos predictivos.

2da parte: hipótesis y objetivos (capítulo 2)

3era PARTE: Resultados

Capítulo 3

Objetivo 1: Conocer los diferentes procesos de elaboración, almacenamiento y distribución aplicados en la industria de vegetales listos para consumo, identificando factores de riesgo y parámetros del proceso.

Capítulos 4 y 5

Objetivo 2: Desarrollar y aplicar modelos experimentales, basados en los parámetros identificados en el objetivo 1, para el estudio de los procesos de lavado y desinfección de vegetales listos para consumo
Objetivo 3: Evaluar el efecto de los procesos de lavado y desinfección sobre el crecimiento de bacterias patógenas y microorganismos indicadores de inocuidad.
Objetivo 4: Desarrollar modelos predictivos de crecimiento microbiológico en base al efecto del lavado y desinfección de vegetales listo para el consumo.

Capítulo 6:

Objetivo 5: Aplicar los modelos predictivos recopilados y desarrollados, resultado de los objetivos anteriores, con datos obtenidos de situaciones reales de producción industrial de vegetales listos para consumo.

4ta PARTE: Conclusiones y reflexiones finales (capítulo 7)

CAPÍTULO 1

1 Capítulo 1: Introducción general

1.1 Ready-to-eat vegetables

1.1.1 Consumption and production of ready-to-eat vegetables in Argentina and Spain

In recent years, society has changed its consumption patterns, increasing the demand for healthy food products with high organoleptic quality, high nutritional value, that are safe and easy to prepare or consume. Consequently, consumers are increasingly incorporating ready-to-eat vegetables into their diets (Mir et al., 2018; Olaimat & Holley, 2012; Randhawa et al., 2015; M. Uyttendaele et al., 2014; Yousuf et al., 2020). Notwithstanding, the acceptance of this type of products is not the same in all regions of the world.

Ready-to-eat (RTE) vegetables, also defined as IV gamma, pre-cut vegetables, fresh cut vegetables, minimally processed vegetables (MPV) or fresh processed vegetables are products that have undergone operations of classification, washing, peeling, size reduction, packaging, etc. Due to this processes, they are marketed as ready-to-eat products or healthy food commodities increasing proportion of vegetables in diet.

Although Latin America produces a great variety and amount of vegetables and fruits, the industry involved in the processing of RTE vegetables has only recently begun to develop. In countries such as Argentina, Brazil, Chile, Uruguay, among others, fourth-range consumption is restricted to large cities (Alonso & Chiesa, 2009; Rodríguez et al., 2015). On the contrary, as reported by Jiménez & Reverté (2018), Europe is the second-largest market after North America for processed fruit and vegetable products, including RTE vegetables.

In particular, the production, distribution, and sale of this type of products is growing at a significant rate in Spain. In 2020, it was reported that the purchase of IV gamma vegetables grew by 1.7%. This category represented for 1.05% of the total budget allocated to household food purchases (MAPA, 2021).

The marketing of RTE vegetables is also very different in both regions. In Argentina, for example, approximately 60% of minimally processed vegetables are sold through supermarkets, and 35% through the HoReCa (hotels, restaurants, and catering services) distribution channel (del Carmen Rodríguez et al., 2017). By the contrary, when displayed in supermarkets, these products do not extend over 10% of an entire shelf of fresh vegetables. In Spain, the main sales channels for these products are supermarkets and self-services, where the products are displayed in entire gondolas, with a market share of 41.2% (MAPA, 2021).

There are no precise data on the market for RTE vegetable products in Argentina, but it is known that the consumption of vegetables is higher as fresh (first gamma), purchased mostly in fruit and vegetable stores, supermarkets, and fairs. Pre-cut products with varying degrees of conditioning and processing are also frequently found. They are marketed without the adequate packaging or cold chain conditions required for this type of products, and generally do not apply Good Manufacturing Practices (GMP), therefore being considered low quality foods with shorter shelf life (del Carmen Rodríguez et al., 2017; Galizio & Diaz, 2020).

The RTE vegetable sector in Argentina is an emerging industry with strong growth potential due to consumer demand, and also as a business strategy to add value to primary production. As indicated by Galizo Diaz (2020), this provides an excellent opportunity for small and medium-sized producers who, through associations or cooperatives, could reach interesting production scales to meet demand at reasonable prices, incorporating new technologies as well as supplying high quality and reliable products. It is important to highlight that experiences from Spanish industries could be taken to "export" them to Argentine industries, taking into account and respecting the characteristics of each country, but leveraging the achievements of the Spanish Industry in the production of RTE vegetables.

1.1.2 Microbiological safety of ready-to-eat vegetables

As above-mentioned, most RTE vegetables are consumed raw, without further processing by the consumer. This fact stresses the importance of ensuring safety conditions of the final product. Vegetables and fruits are among the fresh foods most frequently implicated in foodborne outbreaks, which have been mainly due to contaminations by *Escherichia coli* O157:H7, *Salmonella enterica* or *Listeria monocytogenes* (Callejón et al., 2015; Castro et al., 2018; European Food Safety Authority (EFSA), 2017; González et al., 2017; Mieke Uyttendaele et al., 2015; Zhang et al., 2020).

Vegetable and fruits contamination by pathogenic microorganisms can occur at many points along the food chain. For example, during primary production, using contaminated irrigation water or with unknown microbiological quality, due to lack of analysis. Soil can also be a contamination factor, due to the presence of animals and manure in the croplands that can be sources of several foodborne pathogens. In turn, when the raw material enters the transformation process, the application of incorrect manufacturing practices can cause direct

or indirect contamination of the transformed product. Likewise, contamination can occur in the last stage of the transformation process during packaging, or later during distribution and storage in gondolas. For such reasons, the microbiological safety of these foods is very important and requires a systematic approach that includes all aspects of the production chain, from "farm to fork". The concepts of Quality and Safety Management Systems in vegetable production will be detailed in this Chapter. In addition, the tools and in force regulations to reduce contaminations and risks associated with the production and processing of vegetables will be presented. In this sense, the use of predictive microbiology models should be considered as a valuable instrument able to anticipate microbiological risks along the food chain of ready-to-eat vegetables, identifying effective control measures and risk mitigation strategies.

1.2 Quality and Safety Management Systems in the production of vegetables

Este trabajo fue publicado como un Capítulo del libro Quantitative methods for food safety and quality in the vegetable industry: *"Quality and Safety Management Systems in the Production of Vegetables"*. S. Cuggino, S. Kopp, R. Novo, A. Pérez Agostini. ISBN:978-3-319-68175-7. Springer. Enero 2018. DOI: https://doi.org/10.1007/978-3-319-68177-1_2.

1.2.1 Food Security in the Production of Vegetables

According to the definition of the Food and Agriculture Organization of the United Nations (FAO), "Food security exists when all people, at all times, have physical and economic access to sufficient safe and nutritious food that meets their dietary needs" (1996). Food safety not only implies the offer, but also the availability of innocuous food, taking innocuousness as the intrinsic attribute of a product to be considered suitable for human consumption. Safe food must be free of physical hazards (bones, stones, metal fragments or any foreign matter), chemical hazards (veterinary drugs, pesticides, toxins from microorganisms, cleaning and disinfection agents) and biological hazards (microorganism pathogens) for the consumer.

In all the stages of the production chain of vegetables, the possibility of having high risk contaminants that affect consumer's health is present, thus reducing the quality of the product or driving to its seizure. The concept food safety refers to the conditions and practices applied in the food environment to prevent food contamination and foodborne illness.

To attain the maximum protection of consumers, it is essential that the concepts of innocuousness are introduced in the whole agricultural production chain, from the production to the consumption. An integrated and systemic planning is crucial "from the farm to the table" in which the producer, manufacturer, carrier, seller and consumer have an essential role in ensuring food quality (FAO and WHO 2003; FAO, IFAD and WFP 2014).

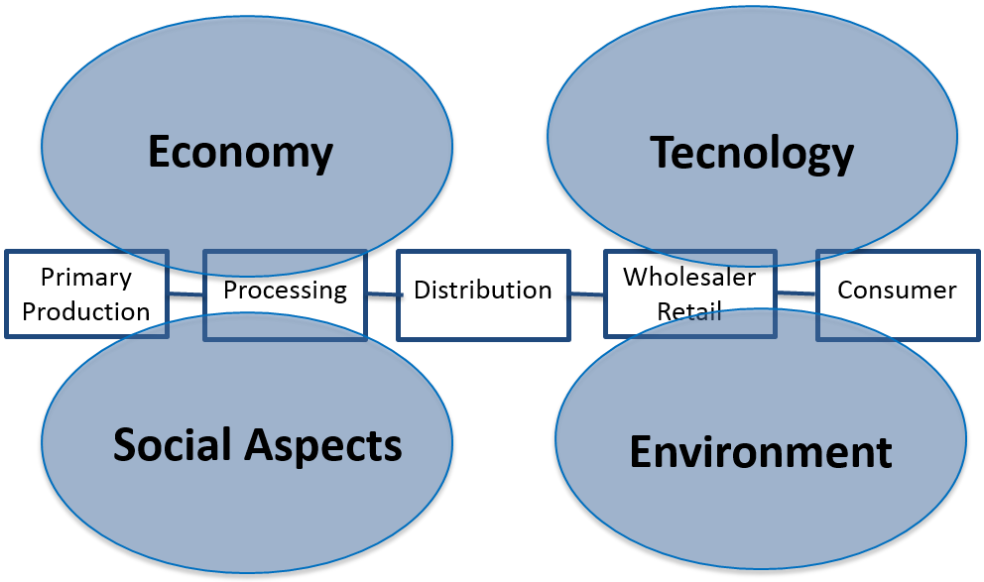

Figure 1.1. Different approaches to the production of vegetables.

When the production chain of vegetables is analyzed as a whole, from the primary production to the final consumer (Figure 1.1), the different focusses that integrate it shall be considered to improve understanding:

- Economic focus: It lets a company to make profit and meet the changing needs of the consumers.
- Environmental focus: It allows for the adequate use of the necessary resources for the production and transformation of food within a sustainable frame.
- Technological focus: It refers to the method of production and transformation of food linked to other steps in the process through technological advances, transport, information and communication to obtain high quality and safe vegetables as a result.
- Social and Legal focusses: Evidenced through the imbalanced growth of some steps in the production chain of agricultural food and to the business barriers that alter the benefit-cost relationship and, last, but not least, the sustainable socioeconomic development.

All the interested parties in the food system, including producers, food transformation and handling, from the production to the storage and final consumption steps, share the responsibility of ensuring safe and nutritious food throughout the whole food production chain. Therefore, it is the responsibility of stakeholders throughout the whole food production chain to adopt a quality management system with an integral focus on food safety. This responsibility also involves an interaction with scientific institutions, legal and normative bodies, as well as social and economic agents, both, at national or international level. Nevertheless, the challenge is to develop integral food systems that ensure long-term involvement and commitment of all the interested parties in order to achieve the desired objective: a safe and nutritious food supply for consumers.

1.2.2 Quality Management System in the Production of Vegetables

A Quality Management System (QMS) is a set of planned actions aiming at attaining consumers' confidence to attain a high quality product or service. The term "Quality Management" refers to four main stages: planning, control, assurance and improvements. All of them are necessary to reach success.

Among the QMSs and the Food Safety used to ensure safe products, more widespread in the production of vegetables, the Good Agricultural Practices (GAP), Good Manufacturing Practices (GMP), the Integrated Pest Management (IPM), Sanitation Standard Operating Programs (SSOP) and the Hazard Analysis and Critical Control Points (HACCP) are applied. Some of them are framed under public rules, both national and international, upon voluntary or obligatory compliance (Figure 1.2).

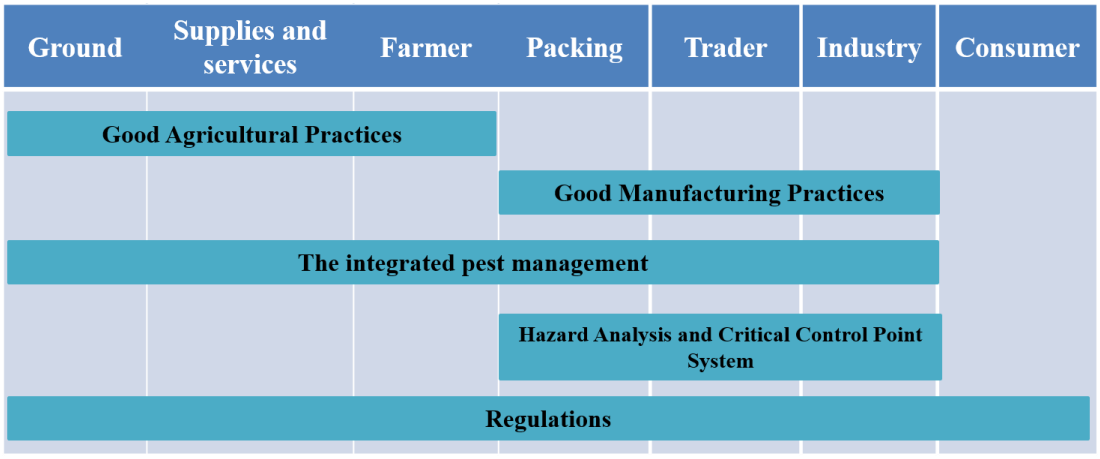

Figure 1.2. Areas of application of regulations.

1.2.2.1 Good Agricultural Practices

The GAP are a set of rules, principles and technical recommendations applied to the different stages of the agricultural production. They include the IPM and the Integrated Crop Management (ICM) which aim at offering an innocuous and high quality product with minimal environmental impact, for the wellbeing and safety of consumers and workers. Furthermore, they enable a framework of sustainable agriculture that can be registered and assessed (FAO, 2014). A simple definition of the GAP is "to do things right" and "to be able to give assurance of this". In this sense, its implementation includes, in each stage, knowledge, planning, recording and management towards the achievement of social, environmental and specific productive goals.

The application of a GAP system is successful when it includes the previous knowledge of the actions and guidelines that rule it, such as: the environment, the sanity and the innocuousness of products, their traceability using records and the safety for workers and consumers.

The application of the GAP is based on the guidelines or manuals that describe the most recommended practices to be applied during the primary production. Particularly, for the production of fresh vegetable for consumption, there are many national manuals that detail the best recommended practices for small as well as large scale producers, in specific environmental conditions.

In European countries, the EUREPGAP standard (1997) applies. Its origins come from the private sector, and it was developed from an initiative of retailers, along with representatives from all the stages of the agro-food production chain and organizations of producers from other parts of the world. In the year 2007, the name was changed for GLOBALGAP, more illustrative due to the protocol's repercussion at international level, together with the third revision of the standard (GLOBALGAP, 2016).

Global compliance with GAP is optional. However, if production is to be marketed in Europe, many clients require compliance with GLOBALGAP standards and its certification. Compliance in these cases becomes a compulsory requirement to operate in that market. Instead, if the buyer is the United States, their application is only recommended.

In Argentina, specifically, the National Food Commission (*Comisión Nacional de Alimentos*, CONAL), in charge of updating the Argentine Food Code (*Código Alimentario Argentino*, CAA), approved in 2008 (RECORD No. 78/2008, ANNEX I) the proposed standard to include the GAP into the CAA for the vegetables, fruits and aromatic sectors and to declare them compulsory as of 2010.

1.2.2.2 Good Manufacturing Practices

The GMP are a means to getting products safe for human consumption, focusing on the hygiene and handling in the food transformation stage.

The scope of the application of the GMP reaches any facility where any of the following activities is carried out: food manufacturing, industrialization, fractioning, storage and transport.

The GMP are applied in:

1- Raw materials
2- Facilities: related to structure and hygiene (SSOP)
3- Personnel

4- Hygiene during manufacturing
5- Storage and transport of raw materials and finished product
6- Production processes control
7- Documentation

In order to take part in many countries of the global market and in the national market, all facilities that wash, pack and label vegetables shall comply with the GMP.
Follow-up of the hygiene conditions can be done through the Sanitation Standard Operating Programs (SSOP) which are well-established and prescribed methods to routinely keep track of safety and hygiene operations performance.

1.2.2.3 Integrated pest management programs

The integrated pest management (IPM) programs use all the available resources through standardized operative procedures to minimize the hazards present in pests. Pest makes reference to any animal that competes with man in the search for water and food, invading spaces where human activities are carried out. Their presence alters the quality of food and they represent one of the most important vectors for the spread of disease, specially food-borne diseases (FBD).
Unlike the traditional pest control (curative), the IPM is a preventive system in response to a plan intended for high quality food. In this way, IPM is a system that allows for fruitful interrelations with other management systems and it constitutes an essential prerequisite for the implementation of HACCP.
IPM can be voluntarily adopted in the primary and industrial production of vegetables. Its application is translated into a planned reduction of the risks of FBD.

1.2.2.4 Hazard Analysis and Critical Control Point System

HACCP is a model with a systemic approach to the identification, assessment and control of practices, processes and spaces in the food production that are critical for product's safety (FAO and WHO, 2015). Currently, the HACCP principles are the basis for most of the quality assurance systems and they involve the following actions:

1. Biological, chemical or physical hazards analysis

2. Identification of critical control points (CCP)
3. Setting of preventive measurements with critical limits for each control point
4. Adoption of monitoring procedures over critical control points
5. Application of corrective actions due to the lack of compliance of any critical limit
6. Implementation of system verification procedures
7. Implementation of an effective recording method to keep track of the HACCP system

HACCP has gained international relevance as an essential tool to ensuring food safety for human consumption (FAO, 2002). Its implementation is compulsory in order to engage in any business transaction in the European Community, while it is only recommended for the US market. In Argentine, there is a standard of voluntary observance (Argentine Normalization and Certification Institute [IRAM] 14104:2001).

1.2.3 Regulations

Demand for perishable foods is no longer restricted to local or regional offering, thus, products interchange has considerably expanded its horizons. These changes have affected production, marketing and distribution of vegetables intended for human consumption. By this, quality emerges as a crucial requirement for marketing. This implies compliance with specifications and certifications imposed by target markets. When analyzing the production, manufacturing and sales sectors of RTE vegetables, rules and regulations of voluntary and non-voluntary application ensure the seamless production in accordance with the environment and that makes an efficient use of resources.

Codex Alimentarius recommendations let governments design and adjust policies and programs within a national control system for food. Thus, the Commission has raised international standards for RTE vegetables establishing specific requirements referred to pesticide residues, microbiologic contamination in food, hygiene and labelling.

Many countries have created specialized agencies or programs intended for ensuring food safety for consumers. In line with this, the European Union has created the European Food Safety Authority (EFSA). The United States has coordinated programs of food innocuousness in charge of federal agencies, such as the Food and Drug Administration (FDA), the Food Safety and Inspection Service (FSIS) and the United States Department of Agriculture (USDA).

Latin American countries have created agencies specialized in food innocuousness in order to increase competitiveness of their products in international markets. Clear examples of this are Chile with the Agriculture and Livestock Service (*Servicio Agrícola y Ganadero*, SAG); Argentina with the National Animal Health and Agri-food Quality Service (*Servicio Nacional de Sanidad Animal y Calidad Agroalimentaria*, SENASA); Brazil with the National Agency for Sanitary Vigilance (*Agencia Nacional de Vigilancia Sanitaria*, ANVISA) and Bolivia with the National Service of Agriculture Sanity and Food Safety (*Servicio Nacional de Sanidad Agroalimentaria*, SENASAG).

Among some compulsory regulations for vegetables, available for the European Union, the following can be mentioned:

- General Food Law - Principle (EC) 178/2002: It establishes the general principles of food legislation and it creates the "European Food Safety Authority". It also determines that compliance with the law is responsibility of the economic operators. It informs the obligatory nature of a traceability system. It forces withdrawal from market of food lots that present health hazards and the reporting to sanitary authorities. Moreover, it sets an alert network for the community.
- Principle 852/2004 relative to the hygiene of food products (H1): its application environment includes the whole chain, from primary production (agriculture and farming) to the industrial stage. In this principle, it is mentioned the compulsory nature of the procedures based on the HACCP and of determining microbiological criteria and requirements relative to temperature according to scientific assessments. The main function is to ensure that imported food meets the same level of hygiene and sanity than local food.

Moreover, there are standards of voluntary application in the different countries. The following are the most important ones in the production of vegetables:

• ISO 22000: Project ISO 22000 "Food Safety Management Systems. Requirements" is an international standard that includes the specific requirements based on the Codex Alimentarius principles of the HACCP system.

• Global Food Safety Initiative (GFSI): Brought by CIES-The Food Business Forum, it includes the world's most important supermarkets and it aims at implementing a scheme of world standards related to food safety and applicable to the whole food production chain.

• British Retail Consortium (BRC): developed by the English supermarkets, it sets requirements for the safety and innocuousness management systems. It requires the adoption of the HACCP system, maintenance of a documented quality management system, and the implementation of GMP and a control system of the product, process and personnel.

• AIB: The American Institute of Baking (AIB) of the United States is a non-profit organization created as a technological center for bakers and food processors in general. It developed its own standards in food manufacturing, production, storage and safety.

1.2.4 Quality Certifications

In the agro-food industry, it is important to define the most valued quality attributes of all consumer groups, its relative importance and its assessment method. Once defined, the main concern of the company is to achieve continuous production and supply of innocuous products with quality levels constantly improving. Quality management systems are developed to keep the set quality standards over time so that the consumer can establish a durable association between the brand or the product and certain level of quality. The compliance of products, processes, services and management systems with the defined requirements in papers so-called technical standards or specifications is assessed by the certification bodies that determine if a company meets the set requirements or not, which lets them gain more prestige among consumers.

Moreover, producers and manufacturers try to set apart from the rest and to stand out by the added value of their products. To this aim, they employ designations of origin and quality labels issued by organizations that adhere to a set of specifications, whose compliance is verified by certifying bodies, credited by public entities.

European legislation allows for three types of certifications: the Protected Designation of Origin (PDO) designates the name of a product of which production, transformation and manufacturing must be done in a specific geographical area, with well-known and proven specific knowledge; the Protected Geographical Indication (PGI) in which the link to the geographic means is still present in, at least, one of the production, transformation and manufacturing stages; and the Traditional Specialties Guaranteed (TSG) that do not refer to the place of origin, but that seeks to highlight a traditional composition of the product or a traditional method of production.

1.2.5 Audits

The audit is a well-organized process of data collection needed to check efficiency of the quality system applied (GAP, GMP, HACCP, ISO, etc.). It is systemic. It includes observation and an on-site review of records to determine if the planned actions are adequate to the ultimate aim of food innocuousness. It is a planned and organized activity based on preset rules and directives due to its formal nature.

Information obtained from the audit is registered in checklists: files that include key points for the execution of the activities. They are presented as forms, surveys or spreadsheets and they represent memory aids that help the auditor follow an organized sequence of observations during the audit. Checklists must be adequate according to the specificity of each audit. They must be simple, objective, easy to use, to read and to understand, and they must identify data and facts.

Audits can be classified as follows:

- *Related to the type*:

Adequacy audit: It is an objective report to check adequacy of a quality system implemented in the facility.

Compliance audit: It is done to check if requirements set in the quality system plan are met in the facility on a daily basis.

- *Related to the company*:

Internal audit: It is done at the initiative and responsibility of the company.

External audit: It is done at the initiative of buyers and the competent sanitary authority of other inspection authorities, among others; not at the initiative of the company itself.

The main purposes that lead to an audit's planning and application are the following:

- To determine compliance or non-compliance of the quality system elements with the specified requirements.
- To provide quantitative results of compliance with the quality management system assessed in the facility.
- To assess the need of improvements or corrective actions based on the non-conformities found.
- To assess efficiency and efficacy of corrective actions.
- To abide by regulatory requirements.

1.2.6 Traceability of Vegetable Products

Traceability can be defined as the documented system that ensures the origin of food and that identifies all the processes and movements of a product from the farm to the table. The main objective of the adoption of a traceability system is food safety to protect and reinforce consumers' confidence.

Traceability in the production chain of minimally processed vegetables must consider the following basic aspects:

a) Products, lots and logic units' identification.
b) Record of all successive stages in the supply chain.
c) Record of relevant information to be traced throughout the chain.
d) Provision of all the necessary information to the next participant in the process to lend continuity.

Traceability systems are characterized by their breadth, depth and accuracy. Breath describes the quantity of information that the system records. Depth refers to the extension of records throughout the whole production chain, including the control period. Accuracy refers to the degree of security with which the system can detect movements or characteristics of the product, assuming certain level of error depending on the size of the defined lot. Thus, availability of information about each product, from the farm to the sales point, is broad, extensive and accurate and it contributes to an efficient quality management in the production chain.

In the food industry, in general, and in the horticultural industry, in particular, with very tight profit margins, the possibility of reducing costs in transport, storage, control and possible seizures of goods contributes to the success or failure of the business activity.

Although traceability systems for themselves do not improve the quality of vegetables intended for direct consumption or minimally processed, they have the ability to identify errors in the process, to assign civil liabilities for the alteration and to test the proper function of the safety systems.

As regards the implementation of a traceability system, the main restraints are the costs associated with the record-keeping. These costs may be reduced by dividing the flow of products in discrete units (lots) taking into account the processes or the set of common attributes, simplifying the distribution system and integrating the suppliers' and distributors' sectors. This allows for coordination between production, transport, processing and

marketing. Each participant in the production chain shall harmonize all the systems present in the control of food safety.

Although records of primary production are kept, at present, traceability applied to the agroproductive system of fruits and vegetables needs a technical recording management system consolidated with professional counselling, if necessary. In the next stages of distribution and sales, a traceability system needs to be incorporated. This should consider the set of possible packages, identification codes, and logistics, and distribution solutions, available in the form of new technologies.

1.2.7 Quality and Non-quality Cost

The adequate application of an integrated system of management practices for the production and transformation of RTE vegetables that encompasses from the primary production to the transport, packaging and marketing pretends to ensure innocuousness and to achieve certain level of product quality. By these means, suitability of food for human consumption is guaranteed, in addition to ability to enter into different markets with laws that include concerns of suitability. The producer and processor that apply the good practices meet the requirements to place their products in foreign markets (more demanding and competitive than ever before). They are also able to distinguish their products in local market.

The production of vegetables that meets quality standards carries evident benefits from the economic and business points of view. Applying an integrated QMS, although it increases some operative costs, it reduces the costs of low quality which ultimately results in a measurable benefit. Not applying QMS leads to a greater increase in costs, mainly those derived from noncompliance with certain attributes imposed by clients. This results in rejected and returned goods or seizures, lost materials and labor, penalties and recharges, among other consequences.

1.2.8 Risks and Control Tools in the Production of Vegetables Using Aa Integrated Quality Management System

In recent years, due to changes in consumption patterns, consumers have increased the demand for food products of high organoleptic quality, healthy, innocuous and that are easy to consume or prepare. This has sped up acceptance of RTE vegetable products. However, these products can present a potential risk for health due to the absence of thermal treatment

in their preparation. It is essential to apply a quality management system based on GAP and GMP, together with microbiological and chemical controls throughout the whole production chain.

1.2.9 Risks Associated with the Production of Vegetables

The concept of risk associated with food tends to show discrepancies between consumers' perceptions and the actual risk (Figure 1.3).

Figure 1.3. Classification of risks for food security as assessed by experts (real risk) and according to consumer perceptions (perceived risk).

Risk analysis is a process made up of three interrelated and integrated elements: risk assessment (RA), risk management and communication of the risk.

Microbial risk assessment is a science-based process intended to determine hazards in food, the probability of exposure to them and their effect in public health. It is based on scientific grounds, on the identification and characterization of hazards, and on the estimate of exposure and characterization of the risk (FAO and WHO, 2009).

Risk management consists in using the collected data and attending to other considerations (technical viability, economic costs, social costs, etc.) in the assessment stage in order to make decisions as regards the need or viability to implement measures. The communication of the risk implies the interactive interchange (throughout the whole risk analysis process) of information and opinions in relation to hazard factors and risks, risk factors and risk

perceptions, established among those responsible for the risk assessment and management, consumers, food industries, the scientific community and other interested parties.

Epidemiologic information is critical to establish the cause that leads to certain food related disease, apart from other factors involved. It is expected that epidemiologic information lets confirm the existence of a pathogenic-food-host link. This is the first stage of RA: risk identification.

The result of a RA will ground the decision of risk managers to set certain Food Safety Objective (FSO). FSO is defined as "the maximum frequency and/or concentration of a hazard in a food at the time of consumption that provides or contributes to the adequate level of protection" (ICMSF 2002; CAC 2013). The objective of FSO is to translate an adequate and tolerable level of protection for the consumer in attributes measurable by the food industry, so appropriate control measures can be implemented.

Although the FSO is the chosen tool for the design and control of operations in the food industry, it does not pretend to verify the acceptance of a lot. However, a FSO can be used as a basis for setting microbiological criteria.

A microbiological criterion must be set and applied when actual need exists and when its application is deemed practical. Such a need is shown: (1) by epidemiological evidence that the food concerned represents a risk for public health and that certain approach is of utmost importance for the protection of the consumer, or (2) as a result of a RA.

A microbiological criterion defines the acceptability of a food, a lot or a process based on the absence, presence or the number of microorganisms and/or its toxins/metabolites per unit of volume, surface, or lot.

Microbiological criteria related to food involve:

- A description of microorganisms and/or its toxins/metabolites and the reason of interest.
- Analytical methods of detection.
- A plan that defines the number of field samples to be taken.
- The magnitude of the analytical unit.
- Microbiological limits considered appropriate for food in specific point(s) of the food chain.
- The number of analytical units that must adjust to these limits.

- The food to which they apply.
- The points in the food chain.
- The measure to be taken when this criterion is not followed.

The *Codex Alimentarius* regulates the application of the microbiological criteria through a set of Food Standards globally adopted, uniformly presented and taken as referential in the resolution of business problems by the World Trade Organization. The aims of these standards are to protect consumers' health and to enable global trade of food. They pretend to serve as a guide and to encourage production, manufacturing and consumption of innocuous food.

1.2.10 Microbial Contamination in Vegetables

Vegetables have the potential to host pathogens, such as: *Salmonella* spp, *Listeria monocytogenes*, *Shigella* spp, *Clostridium botulinum*, *Escherichia coli*, *Campylobacter* spp, *Yersinia* spp, *Vibrio* spp and *Staphylococcus aureus* (FAO and WHO, 2008), *Cryptosporidium* and Hepatitis A virus, fecal contamination indicators, i.e. *E. coli*; which are spread by food intake. As regards canned vegetables, the most common contaminants are *Bacillus* spp and *Clostridium* spp.

Contamination of vegetables may be superficial or it may go deeper into inner tissue. When these microorganisms are present in vegetables, they are able to survive in different surfaces, to overcome stress conditions and to remain latent until the right conditions to grow and colonize are present.

FBD are caused by the intake of contaminated food and/or water in quantities enough to damage the consumers' health. They can be divided into infections and toxicological infections depending on the nature of the contamination: by microorganisms or by the toxins they produce, respectively.

1.2.11 Reduction of Microbiological Risks in Vegetables

At present, different technologies and treatments are used to control microbiological risks, i.e. disinfection, thermal treatment, edible coating, a modified atmosphere and refrigeration to extend shelf-life of these vegetables.

The most widely used processes in vegetable sanitation can be classified as follows: physical methods, such as washing under high-pressure water at low temperature (4º C); soft thermal treatment (water at 40-45º C); ionizing radiation; UV light application, among others. Some examples of chemical methods include washing with sodium hypochlorite, acetic acid, hydrogen peroxide, peroxiacetic acid, chlorine dioxide, electrolyzed water and ozone.

The washing stage is the most relevant part in the ready for consumption vegetable processing because its utmost aim is to reduce leftover dirty and microbial contamination (pathogenic and altering types) in food. At the same time, water used is the main vehicle for the spread of pathogenic microorganisms. All currently effective international regulations for the production of vegetables demand that the water used along the whole production chain meets sanitary and safety standards.

It is important to highlight that during the packaging of RTE vegetables, the microbiological quality of the product must be reassured. To this end, packages with modified atmosphere can be employed, while keeping vegetables' cold chain during storage and transport. Moreover, the application of combined treatments of antimicrobial coating is been under consideration.

In the particular case of canned vegetables, sterilization and fast cooling are the most effective methods for reducing microbial contamination.

1.2.12 Microbial Detection Methods in Vegetables

Both quantitative and qualitative microbial detection methods mirror the application of quality management systems in the production chain of any food to assess acceptability. None of the commonly used methods let determine the exact number of microorganisms present in a piece of food.

Application of appropriate analytical methods, such as investigation or count of colony forming units or the Most Probable Number (MPN), is proposed by official bodies and international regulations in consideration of their ability to determine or quantify the microorganisms concerned. Methods to be used must have statistically-proven reliability based on comparative studies. Tolerance limits for each type of microorganism, their characteristics and their implications are also defined.

To ensure reliability, data obtained shall be certified in an accredited laboratory, validating results of each microorganism.

On the other hand, the industry may observe microbiological specifications to check compliance with regulatory requirements, to establish design requirements, and to assess products as a means for checking or validating efficacy of their Good Hygienic Practices (GHP) and HACCP. These criteria can be stricter than those imposed by regulatory authorities and they are not applied with regulatory purposes.

1.2.13 Chemical Risk

Pesticide residues in food represent a risk of chemical contamination of vegetables that worry a large fraction of consumers. The development of quantitative analytical techniques favor detection of extremely small concentrations of pesticide in food which were used or previously used in crops.

Pesticides are chemical or biological substances designed to control biotic agents negative for the agricultural production, such as insect pests, dust mites, fungi, bacteria, nematodes, mollusks, rats, among others. Products like plant growth regulators, pest attractants and repellents or ectoparasiticides are also considered pesticides. Pesticide residues are defined as “any substance present in a food product intended for human and animal consumption, as a consequence of a pesticide use” (FAO and WHO, 2015). This definition no only includes the original product, but also its metabolites with toxicological importance, components of the formulation (coadyuvants, inerts, etc.) and factory impurities.

Due to the biocide nature of pesticides, their presence in crops is the reason why many people are opposed to their use. Moreover, lack of information available generates confusion, thus affecting the precautions that consumers may take.

Studies done have shown that these residues generate a minimal, though not null, risk for consumers. Levels of sensitivity to different residual chemical principles vary according to people's genre, age, race. Prolonged exposure to very small quantities of toxic residues may produce long term effects on health, especially carcinogenic effects.

1.2.14 Maximum Allowable Limits and Regulations

The most important importing countries set requirements as regards the maximum residue limits (MRL) of pesticides residues that may remain in the different food products. These

limits are based on national and international rules. Government agencies in the importing countries take samples of products to ensure they do not exceed the preset values. Producers are only allowed to use biological agrochemicals and products that are approved for use in a specific product and strictly following the package specifications.

The Codex Committee on Pesticide Residues is in charge of controlling pesticide residues in food. The main tasks are based on scientific approvals from the group of independent experts, the Joint FAO/WHO Meeting on Pesticide Residues. There follow the functions of the Committee on Pesticide Residues:

- To set maximum limits on pesticide residues.
- To make lists of priorities in relation to pesticides which will be assessed by the Joint FAO/WHO Meeting.
- To inspect sampling and analytical methods to determine the presence of pesticide residues in food.
- To set maximum limits of environmental and industrial contaminants that have characteristics similar to those of pesticides.

Since the Uruguay Round of the General Agreement on Tariffs and Trade (GATT), Codex has laid the foundations of international food standards for trade among countries member of the World Trade Organization (WTO). National standards are based on data of a country about toxicology and pesticide residues in crop. Developing countries that have not yet set their own limits can use the residue limits recommended by Codex. The intention is to reduce to a minimum the adverse effects that sanitary and phytosanitary regulations may have on the trade of agricultural products. All this is established in the Sanitary and Phytosanitary (SPS) Measures Agreement of this body.

1.2.15 Legislative Alignment in Relation to MRL

There is high variance in the MRL set in the different countries for the same product and food. This builds barriers for agricultural and farming products trade or it constitutes no-tariff barriers to trade. This shows the evident need of a legislative alignment in relation to MRL. In response to this problem, legislative alignment in carried out within the WTO under the SPS Measures Agreement and the Agreement on Technical Barriers to Trade (TBT). Table 1.1 shows how MRL varies from country to country for the same pesticide in apples. This

illustrates the need of legislative alignment based on the Codex requirements in order to streamline the movement of food and to inform the consumer.

Table 1.1. Differences between countries MRLs for the same pesticide in apple [1].

Pesticide	Argentina MRLs	EU MRLs	Rusia MRLs
Azinphos-methyl	0.5	0.05	2.0 Codex
Chlorpyrifos	0.2	0.5	0.005
Mercaptotion	0.5	0.05	0.5
Phosmet	5.0	0.2	10 Codex
Acetamiprid	0.5	0.1	0.03
Thiacloprid	0.5	0.3	Not allowed
Novaluron	2.0	2.0	3.0 Codex
Methoxyfenocide	0.5	2.0	2.0 Codex
Spinosad	0.2	1.0	0.1 Codex
Lambdacialotrine	0.2	0.1	0.03
Bifenthrin	0.5	0.3	0.04

[1] Pesticide MRLs are expressed as ppm

Source: INTA EEA Alto Valle (2008)

1.2.16 Detection Method for Chemical Residues in Vegetables

For a correct analysis of residues, a highly representative sample of material must be obtained. For example, for fruits and vegetables (heterogeneous products made up of units), the size of the sample will depend on the weight of each unit.

Zero tolerance for certain pesticide means that no "detectable" quantity of pesticide must be present in the sample. This is a relative concept since its determination depends on the "detection limit" of the analytical method used. *Codex Alimentarius* does not use the concept zero tolerance. Instead, the MRL set is very low, in consonance with the current detection limit. The detection limit is, then, the lowest concentration of residues of certain pesticide that can be measured with an acceptable grade of certainty using analytical methods officially approved.

The analytical methods employed to determine the concentration of pesticide residues in food are very different depending on the specific food and pesticide being analyzed. Routine methods are called multiresidue because they have the potential to determine an extensive number of pesticides in many types of food. In general terms, they imply four stages: extraction, cleaning or "clean up", determination and validation of results. In Table 1.2, the different techniques used in each stage are mentioned.

Table 1.2. Stages and Techniques in multi-residue analysis.

EXTRACTION	Acetonitrile Acetone Ethyl acetate
"Clean Up"	Liquid-liquid partition HPLC SPE
DETERMINATION	GC-ECD, NPD, FPD, MS HPLC-UV, FL
CONFIRMATION	GC-MS HPLC-MS Application of a second method

In Table 1.3, the techniques used to determine particular groups of pesticides are mentioned.

Table 1.3. Techniques used for each type of pesticide.

Gas Chromatography (GC)	
GC-ECD	Halogenated Pyrethroids Chlorophenoxy acids Phenylurea
GC-NPD	Phosphorus (P mode) Sulfur (S mode)
GC-ITMS/MSD	Most analyzed by GC
High Resolution Liquid chromatography (HPLC)	
HPLC-FL/UV	N-methylcarbamate (RPC-OPA / FL) Phenylurea (RPC-OPA / FL) Acids Clorofenoxicos (UV) Benzimidazoles (UV / FL)
No Chromatographic Methods	
Calorimetry	Dithiocarbamates

1.2.17 Final Remarks

Concepts, tools and regulations mentioned in this chapter contribute to the development of a new consumption culture. In this way, it would be possible to promote food reeducation including not only aspects of nutritional value through the incorporation of vegetables to the diet, but also necessary considerations about innocuousness and conscious assessment of quality attributes that outline a different consumer who demands, on the one hand, low-cost products and, on the other, efficiency on the production system and food transformation in a sustainable framework in relation to resources.

1.3 Predictive Microbiology

1.3.1 Historical remarks

Predictive microbiology is a specialized field of food microbiology dedicated to studying and predicting microbial behavior in foods. This discipline underpins on the premise that microbial response to environmental factors is reproducible and that, by identifying relevant factors in foods, it is possible to predict microbial dynamics in other similar food environments (Ross et al., 2014; Valdramidis, 2016). To this end, predictive microbiology combines classical microbiology with mathematical modeling and statistics.

Mathematical functions are built considering the relationship between microbial dynamics and intrinsic (pH, aw, competing microbiota, etc.) and extrinsic factors (temperature, atmosphere, humidity, etc.) and/or the food processing conditions (Pérez-Rodríguez & Valero, 2013).

Predictive microbiology concepts are not new as there is reference to their use in the literature of 1922, when Esty & Meyer (1922) proposed the model of "Botulinic Cooking". It describes the minimum time of a heat treatment at 121°C applied to canned food with a pH ≥4.5 to achieve a 12-fold reduction of spores of Clostridium botulinum type A (McMeekin & Ross, 2002; Whiting & Buchanan, 2001).

Subsequently, in the 1980s, predictive microbiology undergone a great boost, due to a major concern of industry and government on microbial food safety and the technological advances in computer science and statistical software facilitating calculations required to build predictive models (Herrera, 2013; Membré & Lambert, 2008).

In recent years, there has been a considerable of predictive microbiology as a fundamental tool to support decision-makings in the field of food quality and safety. Indeed, such models can provide a rapid and reliable response to specific microbial safety questions. From a risk analysis perspective, food safety policy should be supported by outcomes from (quantitative) risk assessment studies, which are strongly linked to the application of predictive models in the field of microbial food safety (Pérez-Rodríguez & Valero, 2013).

1.3.2 Model classification

Predictive models can be classified according to different criteria, which are not mutually exclusive. Based on the type of data and biological basis used for model building, models can be classified as empirical or mechanistic. Empirical model are mathematical functions describing observations obtained from fitting or regression processes. However, mechanistic models are developed on a better knowledge of the biological mechanisms governing microbial response (McLauchlin et al., 2004). Models can be also classified according the phenomenon to be described. Thus, kinetic models are intended to account for microbial levels changes (i.e., death and growth) over time. In contrast, when models are based on the probability of a specific event may occur in a given microbial population (e.g., toxin production, metabolic activity, etc.), models are so-called probability models. The latter type can be applied to predict the probability of growth under specific environmental conditions within a certain time lapse. Also, these models are intended to define growth-non growth boundaries within a variable space. In other words, they depict the combination of certain factor values defining the transition zone between growth and non-growth conditions. There is another type of classification, based on the level of description and development, giving rise to primary, secondary or tertiary models (Whiting & Buchanan, 1993).

Primary models are mathematical expressions that describing changes in microbial concentrations as a function of time, using a limited number of kinetic parameters (initial concentration, lag time, growth rate, and maximum population density) (Dalgaard & Mejlholm, 2019; McKellar et al., 2004). This category includes growth, inactivation and survival, models (McKellar & Lu, 2004).

The model by Baranyi & Roberts (1994) is the most widely used, as it includes a pseudo biological parameter, representing for the physiological stage of the microbial population and

can be applied under dynamic environmental conditions. Moreover, the model can be easily fitted through an Excel add-in developed by the model author (DMFit, Institute of Food Research, Norwich, UK).

Secondary model describes the effect of environmental conditions (physical, chemical and/or biological) on the kinetic parameters of a primary model (Ross & Dalgaard, 2004). The most widely used secondary models are the polynomial ones which corresponds to the so-called response surface models (RSM) (J. Baranyi et al., 1996; McClure et al., 1993), and square root models (Ratkowsky et al., 1982).

Tertiary models are the joint applications of one or more primary and secondary models. This can be performed in dedicated computer software which facilitates its use by non-expert users. Users can perform predictions of microbial growth, survival and death defining different environmental conditions in food by using an easy-to-use interface (József Baranyi & Tamplin, 2004).

It is worthy to note that the rapid development of the software engineering over the last decade has favored the design and implantation of some very useful software tools in predictive microbiology, exhiting a wide range of uses (Pérez-Rodríguez & Valero, 2013). The MicroHibro software, developed by the HIBRO research group of the University of Córdoba in Spain, can be mentioned as an example of the above-mentioned. This tool allows for evaluating the response of pathogens and spoilage microorganisms along the food chain, through estimates of the level of exposure at any point of a food process alongside the attendant risk. The application is underpinned on an extensive database of predictive microbiology models. In addition, MicroHibro allows different predictive models to be compared and validated by entering user data (González et al., 2019; Tenenhaus-Aziza & Ellouze, 2015).

Due to the wide variety of applications of predictive microbiology being available nowadays, their use by the industrial sector have become more and more frequent, undergoing a significant increase over the last few year (Table 1.4).

Table 1.4. Applications of predictive microbiology related to the food industry (McMeekin & Ross, 2002; Membré & Lambert, 2008).

Applications of predictive microbiology
Hazard Analysis and Critical Control Points (HACCP)
- Preliminary hazard analysis - Identification and establishment of critical control points - Corrective measures - Evaluation of the relationship between of variables
Risk Assessment and Management
- Estimation of the dynamics of microbial populations throughout the food chain - Exposure assessment of specific pathogens - Design of scientifically based management strategies to ensure food safety
Useful Life Studies
- Growth prediction of pathogens and spoilage microorganisms in foods
Hygienic Measures and Temperature Integration
- Impact of the cold chain on microbial deterioration - Optimization of inactivation and non-thermal processes
Education
- Education of scientific and non-scientific personnel - Implementation and training of computer-based decision systems
Experimental Design
- Estimation of the number of samples for predictive microbiology experiments - Definition of intervals within each factor to be analyzed
Innovation and Development of a New Products
- Impact of microbial deterioration on product shelf-life - Effect of operation units on food quality and safety - Evaluation of the effect of other additional factors/ingredients throughout the food chain
Operational Support
- Supporting food safety decisions when improving or executing a process - Establishment of critical points and evaluation of effect of deviations on the safety and quality of food
Incidence Support
- Estimation of the impact on consumer safety or product quality in the event of any anomaly in the market

Even though predictive microbiology has a wide variety of applications, bridging the gap between scientific development and practical implementation in the food industry remains a challenge in countries like Argentina.

1.3.3 Modeling cycle

Below is a description of the modeling cycle described by Valdramidis (2016), detailing the steps necessary to develop predictive microbial models (Figure 1.4).

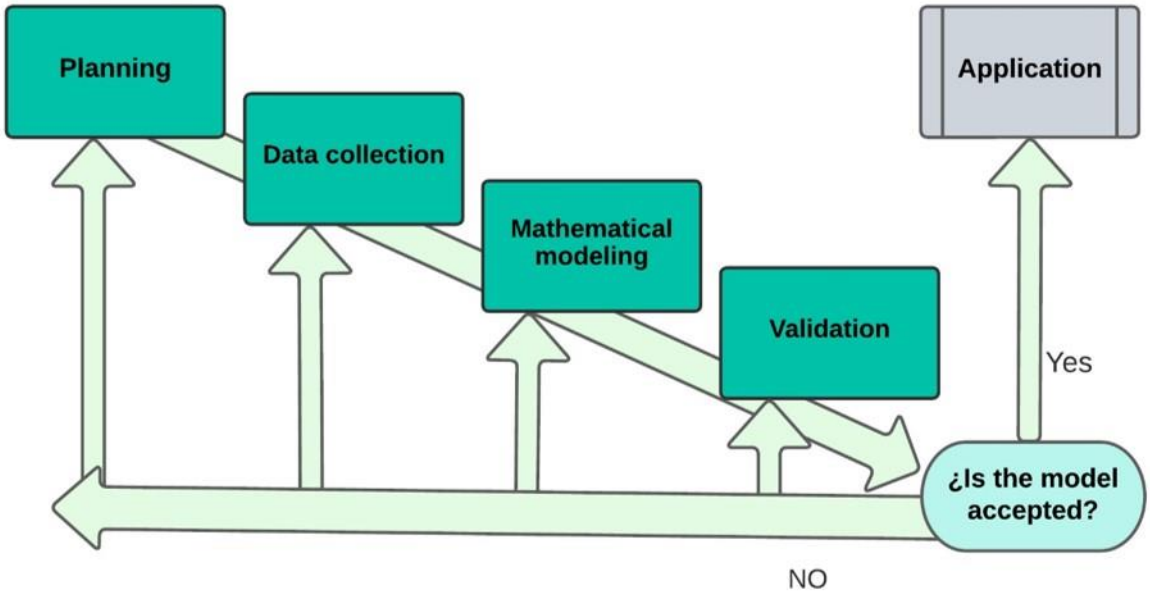

Figure 1.4. Modeling cycle (Valdramidis, 2016).

1) Planning: This step involves defining the problem and specifying the objective of the model. As Draper and Smith (1998) indicated: "The specific statement of the problem is the most important phase of any problem-solving procedure". In this phase, the compilation of previous microbiological knowledge that can explain and/or simplify the observations of microbial behavior is carried out. In addition, the experimental objective, the variables or environmental factors to be analyzed and their ranges are defined as well as the inoculum and the matrix to be used, which can be a food or an artificial broth (Costa & Kristbergsson, 2009; Kilcast & Subramaniam, 2000; Mcdonald & Sun, 1999). Also, it is important to determine the possible influence on or competition with other microorganisms or with the native spoilage microbiota of food.

2) Data collection: Predictive models can be built with existing or new generated data. Experimental data can be obtained from plate counts, microscopic observations, or optical density (i.e. indirect measures) (Mertens et al., 2012).

   In cases where more than one environmental factor is involved, multifactorial designs are developed (complete or fractional factorial experiments, central composite designs, among others).

   Existing data, for example, can be obtained from the national alert systems or monitoring systems. For example: the Rapid Alert System for Food and Feed (RASFF, https://webgate.ec.europa.eu/rasff-window/screen/search), which is the main online database on food safety used by authorities, industry, and scientists in Europe to communicate food safety issues across Europe. In Argentina, there is the ANMAT Food Alerts and Withdrawal system (https://www.argentina.gob.ar/anmat/alertas/alimentos).

3) Mathematical modeling: in this step, one or several appropriate mathematical expressions describing experimental or literature data are selected. First, it is necessary to conduct an exhaustive review of the literature and make a preliminary selection of the primary models. Thus, the kinetic parameters (maximum specific growth rate, adaptation phase, generation time, maximum population density, etc.) for each combination of environmental factors are obtained (Kilcast & Subramaniam, 2000). Then, the adjustment to the secondary models is performed, in order to predict how these growth parameters vary depending on the changes in one or more environmental, extrinsic or intrinsic factors. The main consideration for the selection of mathematical expressions is the accuracy of the fitting, which can be done by linear or non-linear regression methods depending on the character of the applied model.

4) Validation: Predictive microbiological models must undergo validation prior to their application in decision-making. This implies comparing the model predictions with the experimental observations, which can be done internally or externally. Internal validation evaluates the model ability to reproduce observations generated under the same conditions that were used for its development. External validation uses new data, obtained from new experimental conditions, or those reported by other

investigations. If the model is suitable, it can be applied, otherwise the modeling cycle must be repeated re-evaluating the decisions made at the different stages.

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CAPÍTULO 2

2. Capítulo 2: Hipótesis y objetivos

2.1. Hipótesis

En los últimos años, debido a los cambios de los patrones de consumo que ha experimentado nuestra sociedad, se ha incrementado la demanda de productos alimenticios de alta calidad organoléptica, saludables, seguros y que presenten facilidad de consumo o preparación; lo que ha acelerado la aceptación de los productos de IV, como los vegetales listos para consumo. Por tal motivo, y por su potencial riesgo para la salud por la falta de tratamiento térmico, son producto de mucho interés para ser estudiado. El lavado es una de las etapas de mayor relevancia en el procesado de elaboración de vegetales listos para consumo, cuya finalidad principal es eliminar restos de suciedad en el producto, a la vez que reducir la contaminación microbiana en el mismo. En algunos casos, los procesos de lavado llevan asociados un paso de desinfección, para el cual se añade un agente oxidante como el hipoclorito. Estos procesos son altamente dependientes del tipo de agua, de la concentración del desinfectante, de la temperatura del agua y del pH. Un conocimiento más exhaustivo de estos factores, su efecto, y la aplicación de modelos de microbiología predictiva, permitiría desarrollar medidas de control alimentario más efectivas, proporcionando base para identificar factores físico-químicos y parámetros de proceso más adecuados para garantizar la seguridad alimentaria del producto final.

2.2 Objetivos

General

Desarrollar modelos predictivos para el estudio de los procesos de lavado y desinfección de vegetales listos para consumo, identificando combinaciones de parámetros más efectivas sobre la calidad y seguridad del producto final.

Específicos

1) Conocer los diferentes procesos de elaboración, almacenamiento y distribución aplicados en la industria de vegetales listos para consumo, identificando factores de riesgo y parámetros del proceso. (Capítulo 3).

2) Desarrollar y aplicar modelos experimentales, basados en los parámetros identificados en el objetivo 1, para el estudio de los procesos de lavado y desinfección de vegetales listos para consumo. (Capítulos 4 y 5).
3) Evaluar el efecto de los procesos de lavado y desinfección sobre el crecimiento de bacterias patógenas y microorganismos indicadores de inocuidad. (Capítulos 4 y 5).
4) Desarrollar modelos predictivos de crecimiento microbiológico en base al efecto del lavado y desinfección de vegetales listo para el consumo. (Capítulos 4 y 5).
5) Aplicar los modelos de microbiología predictiva recopilados y desarrollados, resultado de los objetivos anteriores, con datos obtenidos de situaciones reales de producción industrial de vegetales listos para consumo. (Capítulo 6).

CAPÍTULO 3

3. Capítulo 3: Evaluación del proceso de elaboración de vegetales listos para consumo en una empresa e identificación de factores de riesgo y parámetros aplicados.

Parte de los resultados obtenidos de este capítulo fueron publicado en:

- NEXO AGROPECUARIO. Vol. N 7. N 1. Julio 2019. *"Optimización del proceso de elaboración de rúcula lista para consumo".* Cuggino, S.; Bressano, M.; González, C.; Mondino M. y Kopp S. ISSN 2346-9110. https://revistas.unc.edu.ar/index.php/nexoagro/article/view/26054.
- Presentación en póster en la VIII Jornadas Integradas de Investigación, Extensión y Enseñanza de la Facultad de Ciencias Agropecuarias 2019: *"Evaluación del proceso de elaboración de rúcula lista para consumo".* Cuggino S., Bressano M., González C.A., Mondino M. R. y Kopp S.B. Córdoba. Argentina.

3.1 Resumen

La aceptación por parte de los consumidores de los vegetales mínimamente procesados (VMP) ha aumentado debido a los cambios en los patrones de consumo. La seguridad microbiológica de estos alimentos radica en la calidad de la materia prima, la eficacia del lavado, la desinfección y el control de la contaminación cruzada durante toda la cadena de producción. El objetivo general de este trabajo fue caracterizar en una industria argentina el proceso de elaboración de vegetales listos para consumo, evaluar el cumplimiento de las BPM y llevar a cabo un plan de muestreo para evaluar la calidad microbiológica de los puntos críticos. De acuerdo con los resultados obtenidos, los puntos considerados críticos para la inocuidad de los productos elaborado fueron: la calidad inicial de la materia prima, la superficie del equipo de corte y de centrifugación, los parámetros del proceso de lavado y desinfección, la calidad del agua utilizada, la limpieza de manos de los operadores y las condiciones de almacenamiento de los productos terminados. Mediante la identificación y la determinación microbiológica de esos puntos, fue posible desarrollar una propuesta de mejoras factible para la industria evaluada. Además, la ejecución de los cambios propuestos dio como resultado una mejora en la calidad microbiológica del producto final y un protocolo de procedimiento optimizado, el cual que puede ser implementado en otras empresas de similares características.

Palabras claves: Calidad microbiológica, Buenas Prácticas de Manufactura, Vegetales listos para consumo, puntos críticos.

3.2 Introducción

En los últimos años nuestra sociedad ha cambiado sus patrones de consumo y ha incrementado la demanda de productos alimenticios de alta calidad organoléptica, saludables, seguros, frescos y que presenten facilidad de consumo o preparación. Esto ha acelerado la aceptación por parte de los consumidores de los vegetales mínimamente procesados (VMP) o listos para consumo (Mir et al., 2018; Randhawa et al., 2015); cuya definición, descripción y criterios microbiológicos están detallados en los Artículos 925 tris y 925 quater del Capítulo XI del Código Alimentario Argentino (CAA, 2017).

Como la mayoría de los VMP no requieren tratamiento adicional antes del consumo, la ausencia de un paso de eliminación de patógenos transmitidos por los alimentos puede resultar en un posible problema de salud pública. De hecho, las ensaladas de hojas verdes se encuentran entre los productos frescos más frecuentemente implicados en brotes transmitidos por alimentos, que se han relacionado principalmente con la contaminación por *Escherichia coli* O157: H7 o *Salmonella* spp. (Callejón et al., 2015; Chaves et al., 2016; EFSA, 2017; Uyttendaele et al., 2015).

La seguridad microbiológica de estos alimentos radica en la calidad de la materia prima, en la eficacia del lavado, la desinfección y el control de la contaminación cruzada durante toda la cadena de producción (Beharielal et al., 2018; Castro-Ibáñez et al, 2016; Koseki, 2014). La trazabilidad de los vegetales de hoja a lo largo de toda la cadena de producción es un elemento esencial para garantizar la seguridad de los alimentos. Por lo tanto, está claro que se requiere una estrategia multidisciplinaria y la aplicación de buenas prácticas de manufactura (BPM) integrando el concepto de "la granja a la mesa" (Gil et al., 2015).

Varios autores han estudiado y evaluado la aplicación de BPM en los procesos de elaboración de vegetales listos para consumo. En estos trabajos los métodos de lavado y desinfección fueron identificados como los pasos más críticos que afectan la calidad, la seguridad y la vida útil del producto (Castro-Ibáñez et al., 2017; López-Gálvez et al., 2009; Meireles et al., 2016; Pérez-Rodríguez et al., 2014). Así mismo, establecer pruebas de evaluación de la calidad microbiológica en diferentes puntos y etapas del proceso de elaboración, es una estrategia adecuada para evaluar la seguridad de los vegetales a lo largo de la cadena de producción (Castro-Ibáñez et al., 2016).

Por ello, a partir de la caracterización del proceso, la evaluación del cumplimiento de las BPM y la determinación de la calidad microbiológica de los puntos críticos en una empresa de vegetales de hoja listos para consumo de Argentina, es posible describir el proceso de elaboración completo e identificar factores y parámetros de riesgo.

3.3 Materiales y métodos

3.3.1 Caracterización del proceso, evaluación de la aplicación de BPM y plan de muestreo

Se seleccionó como objeto de estudio la empresa Ensaladas Prácticas S.R.L., ubicada en Colonia Tirolesa Km. 14 ½, Córdoba, Argentina. El criterio de selección fue que es la empresa de mayor producción de vegetales listos para consumo en la provincia de Córdoba. Además, en la cadena de elaboración de vegetales listos para consumo, abarca todos los eslabones, desde la producción primaria hasta la distribución y comercialización.

En primera instancia, a través de cuestionarios, se evaluaron la aplicación y el grado de cumplimiento de las BPM durante todo el proceso de elaboración de ensaladas de rúcula lista para consumo (AFHORLA, 2010; Garcia y Vazquez, 2015).

Posteriormente, se procedió al diseño de un plan de muestreo de los puntos críticos del proceso a partir del análisis de los datos obtenidos en los cuestionarios y la caracterización de las etapas de elaboración de ensalada de rúcula. Se consideró punto crítico a aquella práctica, proceso, superficie u operación, cuya falta de control o no aplicación correcta resultara en una contaminación, multiplicación y/o producción de microorganismos indicadores de calidad según lo establecido por el CAA (2017) para los VMP.

La toma de muestras de superficies, equipos y operadores fue realizada mediante hisopados. Además, se recolectó materia prima antes de ingresar a la línea de procesamiento, bandejas de ensalada de rúcula al final del proceso y posterior al almacenamiento por 96 h a 7ºC. También, se tomaron muestras del agua de red utilizada, para evaluar su calidad. Todas las muestras fueron almacenadas a 4ºC y transportadas inmediatamente al laboratorio para su posterior análisis.

3.3.2 Análisis microbiológico

Las muestras de hisopados de superficies, equipos y operadores se homogeneizaron en agua de peptona (Britania, Argentina) y se prepararon series de dilución decimal para su posterior

siembra. Con las muestras de agua también se realizaron diluciones seriadas. Para el análisis microbiológico de la materia prima y el producto final, se pesaron 25 g de rúcula y se colocaron en un Erlenmeyer con 225 mL de agua peptona, luego se homogeneizaron por agitación durante 15 minutos y finalmente se realizaron diluciones seriadas para la siembra.
Todas las muestras y sus diluciones fueron sembradas en medios específicos, por duplicado, y se realizó el recuento de microorganismos mesófilos totales, *coli*formes totales, *coli*formes fecales (*Escherichia coli*) y *Salmonella* spp de acuerdo a los protocolos citados en el CAA para vegetales mínimamente procesados (2017).

3.3.3 Diseño y validación del plan de mejoras

De acuerdo con los resultados obtenidos se realizó un plan de mejora basado en las BPM, bibliografía reciente y estudios científicos similares. Posteriormente a la implementación de las alternativas propuestas, se evaluó la calidad microbiológica resultante aplicando la metodología descripta en 3.3.2.
Además, con la finalidad de evaluar la efectividad del tratamiento de desinfección con hipoclorito de sodio (180 ppm) a un pH regulado de 6.5 en relación a la misma aplicación, pero sin regular el pH, se realizaron los siguientes ensayos, simulando las condiciones industriales. Las muestras de rúcula se obtuvieron del campo y se seleccionaron hojas uniformes en tamaño y color y sin daños. Al llegar al laboratorio, las muestras se lavaron con agua del grifo (a ~ 12°C) durante 2 minutos para eliminar las partículas de tierra y bajar su temperatura. A continuación, se cortaron trozos de rúcula con un tamaño de 4x4 cm con una cuchilla estéril y se colocaron en un recipiente de plástico donde se agregó la solución desinfectante (ratio agua/producto: 12 L/kg). EL tratamiento se aplicó durante 6 minutos con constante agitación. Para el tratamiento de desinfección se utilizó hipoclorito de sodio (Ayudin, Argentina) para obtener una concentración final de 180 ppm. El pH se estabilizó en 6,7 con ácido cítrico (Sigma-Aldrich, Argentina). Finalmente, la rúcula fue enjuagada con agua destilada estéril a 8°C por 1 minuto, escurrida y se apartaron 25 g para realizar el análisis microbiológico como se detalló anteriormente.

3.4 Resultados y discusión

3.4.1 Caracterización del proceso, evaluación de la aplicación de BPM y plan de muestreo

La evaluación visual durante el recorrido y el análisis de los cuestionarios permitió realizar una descripción detallada del proceso de elaboración de rúcula lista para consumo llevada a cabo en la industria (Figura 3.1 y 3.2).

Figura 3.1. Diagrama de flujo del proceso de elaboración de rúcula lista para consumo en la Ensaladas Prácticas S.R.L. Izquierda: procedimientos aplicados. Derecha: deficiencia en el procedimiento.

Figura 3.2. Proceso de elaboración de rúcula lista para consumo.

En la Tabla 3.1 se detallan los valores de los parámetros obtenidos del proceso de elaboración de rúcula lista para consumo durante las visitas al establecimiento y su comparación con valores óptimos extraídos de bibliografía (Chen y Hung, 2017; Meireles et al., 2016; Ramos et al., 2013).

Tabla 3.1. Valores de los parámetros del proceso de elaboración de rúcula lista para consumo

Parámetro	Valor óptimo[1]	Valor obtenido
Temperatura de materia prima	<8°C	6,3 °C
Tamaño de Cortado	4x4 cm	4x4 cm
Temperatura del agua de lavado	< 8ºC	17 °C
pH del agua de lavado	6,5-7,5	8,3
Concentración del desinfectante	100-150 ppm	121 ppm
Tiempo de desinfección	180-200 s	360 s
Ratio: agua/producto	< 12 L/kg	12 L/kg
Temperatura de agua de enjuague	< 8 °C	8 °C
Tiempo de secado	-	30 s
Temperatura de almacenamiento	< 8 ºC	7,3 ºC

[1] Valores obtenidos de bibliografía.

El grado de cumplimiento de las BPM aplicadas en la industria fue del 94,2 %. El menor puntaje obtenido se registró en el ítem de Prevención de Contaminación Física, debido a que no cuentan con un sistema de detección de cuerpos extraños. Además, se observó presencia de un vidrio roto de una ventana en la sala de empaque.

Otros de los ítems que no cumplieron con las BPM fueron el del mantenimiento correcto de las áreas internas y equipos, debido a que se observaron algunas paredes sin superficies lisas de fácil limpieza y presencia de algunos equipos con pintura descascarada.

3.4.2 Plan de muestreo y análisis de muestras

De acuerdo con los resultados obtenidos en la caracterización y evaluación del proceso de elaboración de rúcula se diseñó un plan de muestreo de los puntos considerados críticos para la inocuidad de los productos elaborados; estos fueron: la calidad inicial de la materia prima, la superficie del equipo de corte y de centrifugación, los parámetros del proceso de lavado y

desinfección, la calidad del agua utilizada, la limpieza de manos de los operadores y la calidad microbiológica del producto final y posterior al almacenamiento.

El muestreo de los puntos críticos y su siembra en los medios específicos dio lugar a los datos detallados en la Tabla 3.2.

Tabla 3.2. Recuentos microbianos en puntos críticos del proceso de elaboración de rúcula lista para consumo.

Punto de muestreo	**Tipo de muestra**	**Mesófilos totales (UFC/g)**	***Coli*formes totales (UFC/g)**	***Coli*formes fecales (UFC/g)**	***Salmonella spp.***	***E. coli* NMP[1]/g**
Recepción	Rúcula	$2{,}01 \times 10^5$	$4{,}6 \times 10^3$	$9{,}2 \times 10^1$	ausencia	presencia
Cortado	Superficie	$1{,}45 \times 10^3$	0,36	ausencia	No se evaluó	ausencia
Agua de lavado	Agua	$2{,}55 \times 10^3$	ausencia	ausencia	ausencia	ausencia
Desinfección	Rúcula	$1{,}51 \times 10^5$	$9{,}3 \times 10^1$	ausencia	No se evaluó	ausencia
Centrífuga	Superficie	$1{,}82 \times 10^2$	ausencia	ausencia	No se evaluó	ausencia
Envasado	Manos	$1{,}73 \times 10^2$	ausencia	ausencia	No se evaluó	ausencia
Producto final	Rúcula	$1{,}6 \times 10^5$	3,6	ausencia	ausencia	ausencia
Almacenamiento	Rúcula	$9{,}88 \times 10^6$	$1{,}1 \times 10^3$	ausencia	ausencia	ausencia

[1]NMP: número más probable.

Los resultados que se muestran en la Tabla 3.2 indican que el proceso de desinfección aplicado fue eficiente. Logró eliminar la presencia de *Escherichia coli* de la materia prima. Cabe destacar, que la presencia de este microrganismo resalta la relevancia que debe tener el control de los proveedores sobre el agua de riego de la materia prima. En relación a los microorganismos mesófilos y los *coli*formes totales, el tratamiento tuvo permitió disminuir el nivel de la carga microbiológica, pero no eliminarlos completamente.

Se observa, que, si bien las muestras analizadas del producto final no presentaron microorganismos patógenos, cumpliendo con lo especificado por el CAA, éste posee una alta carga de microorganismos mesófilos, lo que redundaría en un mayor deterioro durante el proceso de almacenamiento.

En relación con las superficies evaluadas, todos los valores se encontraron dentro de los parámetros establecidos por el CAA. Contrariamente, el agua utilizada para el lavado de la rúcula no cumplió con los requisitos, dado que se observó un recuento de 2,55 x 10^3 UFC/mL, de mesófilos totales, lo que supera el límite establecido por el CAA (2017) de 0.5 x10^3 UFC/mL. La regulación sugiere que, en el caso de que el recuento supere las 500 UFC/mL y se cumplan el resto de los parámetros indicados, se deberá exigir la higienización del reservorio de agua y un nuevo recuento microbiológico.

En base a los parámetros obtenidos del proceso (Tabla 3.1), la evaluación del grado de cumplimiento de las BPM y la evaluación microbiológica realizada (Tabla 3.2), se identificaron algunos factores de riesgos importantes de resaltar.

El agua utilizada no cumple con los parámetros de calidad requeridos para la higienización y desinfección de la materia prima. Es de destacar que la eficacia de la desinfección final del producto depende de muchos parámetros, como el tipo de desinfectante, la dosificación, la concentración residual, el tiempo de contacto, la temperatura; pero en mayor medida de la calidad del agua de lavado de todo el proceso y la cantidad de materia orgánica en el tanque de lavado (Davidson et al., 2013; Gil et al., 2009; S. Van Haute et al., 2015). Por lo tanto, la higienización del reservorio, a fin de disminuir la carga microbiológica del agua utilizada en el proceso, es una práctica necesaria de realizar.

Además, el control de la temperatura es un punto clave en el crecimiento microbiano. Se puede aplicar refrigeración de agua o del ambiente para controlar la carga microbiana (Meireles et al., 2016). Como se muestra en la Tabla 3.1, la temperatura del agua de lavado y enjuague fue de 17 y 8ºC respectivamente, por lo tanto, es otro parámetro del proceso identificado como riesgoso.

Como lo indican varios autores, la adición de cloro, u otras formas de ácido hipocloroso, en el agua de lavado sigue siendo la práctica de desinfección más aplicada en la industria de vegetales listos para consumo (Meireles et al., 2016; Sam Van Haute et al., 2013) debido a su precio relativamente bajo, facilidad de aplicación y amplio espectro de efectividad antimicrobiano (Chen y Hung, 2017; Guo et al., 2017). Sin embargo, este desinfectante muestra, bajo ciertas circunstancias, una eficiencia limitada para reducir las cargas microbianas ya que puede ser fácilmente inactivado por la materia orgánica (Murray, Aldossari, Wu, y Warriner, 2018; Weng et al., 2016), además de la generación de posibles

compuestos cancerígenos como los trihalometanos que pueden llegar a los consumidores (Gómez-López et al., 2014; Meireles et al., 2016).

Por otro lado, la acción efectiva del cloro es altamente dependiente del pH (Chen & Hung, 2017; Ramos et al., 2013). Por este motivo, se identifica esta práctica del proceso como un factor de riesgo, ya que la eficiencia de desinfección del NaOCl a pH 8,3; como es en este caso, se ve disminuida; y podría mejorarse mediante el agregado de ácido cítrico para regular el pH a 6,5; siendo el valor recomendado (Chen y Hung, 2017; Davidson et al., 2013).

3.4.3 Propuesta y validación del plan de mejoras

Con la finalidad de optimizar el proceso de elaboración de vegetales listos para consumo se propuso a la industria el siguiente plan de mejora: valorar la calidad de la materia prima que ingresa al proceso de transformación, mediante una evaluación de los proveedores o de su producción propia, para conocer la calidad microbiológica y trazabilidad de sus productos. Realizar una limpieza del reservorio de agua y posteriormente un análisis microbiológico. Mantener la temperatura del agua de lavado, desinfección y enjuague por debajo de 8°C. Controlar, regular y mantener el pH del agua de desinfección a 6,5 mediante el agregado de ácido cítrico u otro regular de pH.

Luego de la aplicación de las mejoras propuestas a la empresa se procedió a la toma de muestras para verificar el éxito de su implementación. Los resultados de los recuentos microbiológicos de la materia prima y del agua de lavado reflejaron que su implementación resultó efectiva. La materia prima evaluada no presentó *E. coli* y los valores de mesófilos en el agua de lavado fueron menores a los establecidos por el CAA.

En la Tabla 3.3 se detalla el recuento de microorganismos mesófilos de la materia prima utilizada y de las muestras de rúculas posterior a la aplicación del tratamiento de desinfección con agua y con hipoclorito de sodio (180 ppm), regulando el pH del agua a 6,5 con el agregado de ácido cítrico.

Tabla 3.3. Recuento de microorganismos mesófilos a las 24 horas.

Muestra	UFC/ g de rúcula	Log de UFC/ g de rúcula
Materia prima rúcula	$2{,}01 \times 10^5$	5,30
Rúcula tratada con hipoclorito de sodio (pH=8,3)	$1{,}51 \times 10^5$	5,18
Materia prima rúcula	$2{,}55 \times 10^7$	7,41
Rúcula tratada con agua	$1{,}31 \times 10^6$	6,12
Rúcula tratada con hipoclorito de sodio (pH=6,5)[1]	$2{,}38 \times 10^5$	5,38

[1] pH del agua regulado con agregado de ácido cítrico.

Como se observa en la Tabla 3.3 la materia prima continuó con altos niveles de mesófilos, lo cual indica que se debe profundizar en los controles de los proveedores o prácticas aplicadas en la producción primaria de rúcula. Por otro lado, los resultados muestran que los dos tratamientos redujeron la carga microbiana de la materia prima. El lavado con agua disminuyó en un orden de magnitud la cantidad de microorganismos mesófilos y el tratamiento con cloro (180 ppm) los redujo en dos órdenes de magnitud. Este resultado demuestra la mejora en la eficiencia del desinfectante a base de cloro al regular el pH.

Como resultado final se detalla el esquema del proceso optimizado de elaboración de rúcula lista para consumo (Figura 3.3).

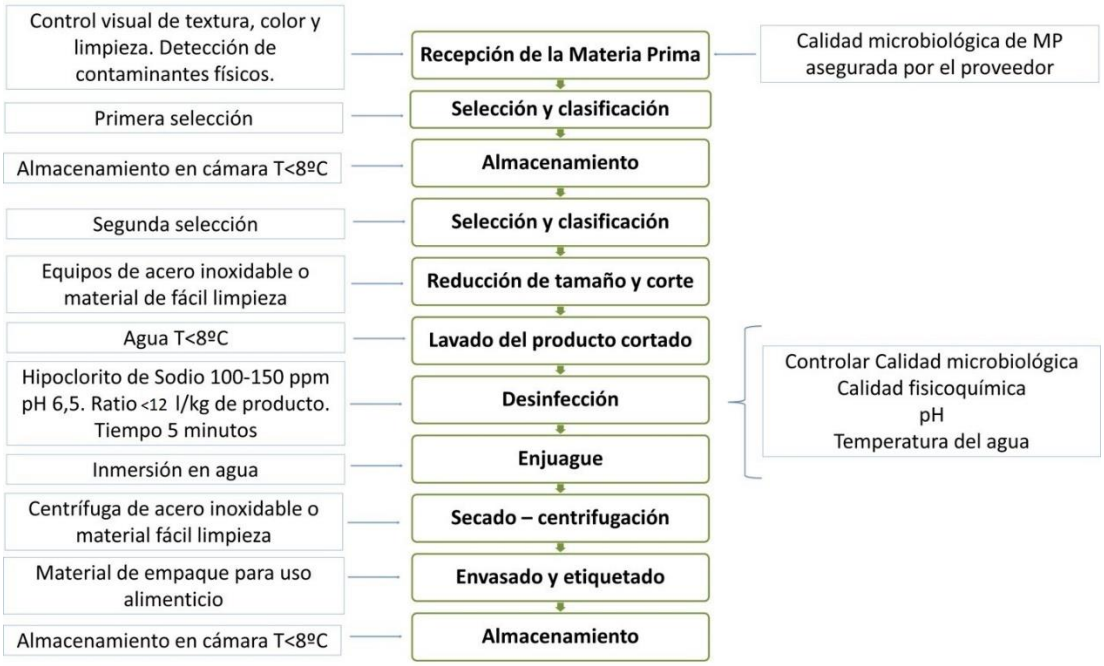

Figura 3.3. Diagrama de flujo del proceso de elaboración de rúcula lista para consumo optimizado

3.5 Conclusión

Los datos recopilados en este estudio permitieron conocer los procesos, parámetros y etapas aplicadas en la producción de vegetales de hoja listos para consumo. A su vez, se profundizó e identificó, basado en estudios de bibliografía, aquellos factores y parámetro del proceso que puedan afectar la inocuidad de los productos elaborados.

Además, mediante la identificación de los puntos críticos de control y la determinación microbiológica en el proceso de elaboración de ensalada de rúcula lista para consumo en la empresa Ensaladas Prácticas S.R.L. fue posible desarrollar una propuesta de mejoras aplicable a la empresa evaluada.

La ejecución de los cambios propuestos dio como resultado una mejora en la calidad microbiológica del producto final y un protocolo de procedimiento optimizado que puede ser implementado en otras empresas de similares características.

Estos datos serán muy valiosos para la aplicación de modelos de microbiología predictiva que se desarrollarán en los próximos capítulos, ya que se basan en información real y pueden utilizarse para evaluar diferentes escenarios de riesgo microbiológico.

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CAPÍTULO 4

4. Capítulo 4: Modelado del efecto combinado de cloro, isotiocianato de bencilo, tiempo de exposición y tamaño de corte en la reducción de *Salmonella* en lechugas fresca cortadas durante el proceso de lavado

Este trabajo fue publicado como artículo científico en la revista Food Microbiology. 2020. Vol. 86, ID: 103346. Titulado "*Modelling the combined effect of chlorine, benzyl isothiocyanate, exposure time and cut size on the reduction of Salmonella in fresh-cut lettuce during washing process*". Con los siguientes autores: S. G. Cuggino, I. Bascón-Villegas, F. Rincón, A. Pérez, G. Posada-Izquierdo, J. Marugan, C. Pablos Carro, F. Perez-Rodriguez https://doi.org/10.1016/j.fm.2019.103346

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Modelling the combined effect of chlorine, benzyl isothiocyanate, exposure time and cut size on the reduction of *Salmonella* in fresh-cut lettuce during washing process

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Los resultados preliminares fueron presentados en el 10 th International Conference on Predictive Modelling in Food, 2019, titulado "*Mathematical assessment of the combined effect of chlorine, benzyl Isothiocyanate, exposure time and cut size on the reduction of Salmonella in fresh-cut lettuce during washing process*". Con los siguientes autores: S. G. Cuggino, I. Bascón-Villegas, F. Rincón, M. A. Pérez, G. D. Posada-Izquierdo, J. Marugan, C. Pablos Carro, F. Perez-Rodriguez.

Además, el desarrollo de este trabajo fue presentado en una conferencia a investigadores de la Facultad de Agronomía, Udelar Uruguay, titulada "Aplicación de la microbiología predictiva en el estudio del impacto de los desinfectantes en la seguridad microbiológica de vegetales mínimamente procesados".

4.1 Resumen

Este trabajo tuvo como objetivo evaluar el efecto de la combinación de hipoclorito de sodio, que es el desinfectante más utilizado en la industria de vegetales, con un antimicrobiano natural, isotiocianato de bencilo (BITC), el tamaño de corte y el tiempo de contacto, en la reducción de *Salmonella* en vegetales listos para consumo, en operaciones de lavado simulando condiciones industriales típicas. En general, las combinaciones de desinfectante y parámetros de proceso dieron como resultado una reducción media de *Salmonella* de 2,5 log UFC/g. Según el análisis estadístico, las concentraciones de cloro libre y BITC, el tiempo de contacto y el tamaño del corte ejercieron un efecto significativo en la reducción ($p \leq 0,05$). La combinación óptima de los parámetros del proceso que produjo la mayor reducción de *Salmonella* fue el tamaño de corte de lechuga de 15 cm^2, la aplicación de la desinfección durante 110 s en agua industrial con 160 mg/L de cloro libre y 40 mg/L de BITC. El modelo predictivo desarrollado podría aplicarse para optimizar el proceso de desinfección industrial y desarrollar evaluaciones de exposición probabilísticas considerando el efecto de los parámetros del proceso sobre los niveles de contaminación por *Salmonella*, en vegetales de hoja. Asimismo, el presente estudio demostró la eficacia del cloro para reducir las poblaciones de *Salmonella* en lechugas precortadas y destacó la importancia de controlar los parámetros del proceso de desinfección estudiados para aumentar la eficacia del desinfectante y mejorar la seguridad alimentaria.

Modelling the combined effect of chlorine, benzyl isothiocyanate, exposure time and cut size on the reduction of *Salmonella* in fresh-cut lettuce during washing process

Abstract

This work aimed to study the effect of the combination of Sodium hypochlorite, the most used disinfectant by the vegetable industry, with a natural antimicrobial, benzyl-isothiocyanate (BITC), considering cutting surface and contact time, on the reduction of *Salmonella* in fresh-cut produce in washing operations under typical industrial conditions. Overall, the combinations of disinfectant and process parameters resulted in a mean reduction of *Salmonella* of 2.5 log CFU/g. According to statistical analysis, free chlorine and BITC concentrations, contact time and cut size exerted a significant effect on the *Salmonella*

reduction ($p \leq 0.05$). The optimum combination of process parameter values yielding the highest *Salmonella* reduction was a lettuce cut size of 15 cm^2 washed for 110 s in industrial water containing 160 mg/L free chlorine and 40 mg/L BITC. A predictive model was also derived, which, as illustrated, could be applied to optimize industrial disinfection and develop probabilistic Exposure Assessments considering the effect of washing process parameters on the levels of *Salmonella* contamination in leafy green products. The present study demonstrated the efficacy of chlorine to reduce *Salmonella* populations in fresh-cut lettuce while highlighting the importance of controlling the washing process parameters, such as, contact time, cut size and concentration of the disinfectant to increase disinfectant efficacy and improve food safety.

Keywords: Predictive microbiology, composite central design, vegetable disinfection, response surface methodology, quantitative microbial risk assessment, sodium hypochlorite, microbial inactivation.

4.2 Introduction

Global consumption of RTE vegetables has continuously increased over the last years (Mir et al., 2018; Randhawa et al., 2015; Uyttendaele et al., 2014). Most fresh produces are eaten raw or after minimal processing without any lethal treatment, enabling that pathogens can be present on produce at the moment consumption. Leafy green products are among the most frequently implicated fresh produces in food-borne outbreaks, which have been related mostly to contamination by *Escherichia coli* O157:H7 or *Salmonella enterica* (Callejón et al., 2015; European Food Safety Authority, 2017; González et al., 2017; Holvoet et al., 2014a, Holvoet et al., 2014; Uyttendaele et al., 2015).

Disinfection is one of the most critical steps impacting quality, safety, and shelf-life of the product (Meireles et al., 2016; Posada-Izquierdo et al., 2014, 2013). Disinfection efficacy depends on many parameters such as the disinfectant agent, dosage, residual concentration, contact time, temperature, pH, wash water/surface ratio where the sanitizer is applied, and the content of organic matter in the washing tank, as well as other physicochemical properties of the process wash water (Posada-Izquierdo et al., 2013; Van Haute et al. 2015). Thus, optimizing these disinfection parameters is key to reduce undesirable outcomes, especially

those that may negatively affect consumer health (Banach et al., 2015; Pérez Rodríguez et al., 2011).

Although chlorine is still the most used disinfectant, due to its efficacy, low cost-effectiveness ratio and simple use, the negative effects due to the generation of possible carcinogenic compounds like trihalomethanes (THMs) that can reach consumers. Moreover, the use of high chlorine concentrations may cause corrosion on stainless steel surfaces and negative sensory effects on product as it leads to unpleasant flavour/odours and produces taints on the vegetable tissue (Gil et al., 2015; Gómez-López et al., 2014; Van Haute et al., 2013). Therefore, one of the main goals of the fresh-cut industry is to reduce and/or replace the use of chlorine. The use of natural antimicrobials, derived from microbial, plant, or animal sources can be considered as a suitable strategy for vegetable disinfection (Pablos et al., 2018) especially when it is combined with other disinfects, which, in the case of chlorine, could help to reduce its concentrations. Isothiocyanates (ITCs) are products originated from the enzymatic hydrolysis of glucosinolates, which are sulphur-containing glucosides present in plants of the family Brassicaceae. For instance, the benzyl-isothiocyanate (BITC) is an isothiocyanate found in plants of the mustard family (*Brassicaceae*). The isothiocyanates have wide-spectrum antimicrobial activity (Aires et al., 2009; Dufour et al., 2015, 2012; Kaiser et al., 2017; Romeo et al., 2018; Sofrata et al., 2011), and are usually regarded as safe (GRAS) compounds; since they are allowed to be in contact with food. The antimicrobial spectrum of isothiocyanates is wide, inhibiting both Gram-positive (L. *monocytogenes* and S. aureus) and Gram-negative bacteria (*Cytophaga*, *E. coli* O157:H7, *Pseudomonas corrugata*, *Salmonella* Montevideo, *Salmonella* Typhimurium, and *Serratia grimesii*) in a concentration range from 0.02 to 200 mg/L (Aires et al., 2009; Dufour et al., 2015; Jang et al., 2010). Authors hypothesized that BITC, having both lipophilic and electrophilic properties, might penetrate through the outer bacterial membrane and hamper the ability of the bacterium to maintain its membrane potential (Romeo et al., 2018; Sofrata et al., 2011). In addition to this, a direct oxidative mechanism can take place, especially upon the reaction with hydrogen peroxide (Dufour et al., 2015).

Predictive microbiology is a scientific field aimed to quantify the microbial behaviour in foods considering the effect of intrinsic (e.g. water activity, pH) and extrinsic (e.g. temperature) factors to develop mathematical models able to predict microbial response

under specific conditions (Pérez-Rodríguez and Valero, 2013). Mathematical models describing disinfection processes for microorganisms on vegetable products, and specially leaf produces are scarce in literature (Pirovani et al., 2004). The development of suitable predictive models for vegetable disinfection can be of great importance for the vegetable industry in order to better understand the effect of the process parameters (contact time, disinfectant levels, etc.) on the disinfection efficiency (Van Haute et al., 2015) and, based on predictions, to optimize disinfection processes. For example, a possible application could be to find process parameter combinations allowing for a lower chlorine concentration while a similar disinfection efficacy is obtained. Besides that, the integration of food-specific disinfection models into the Quantitative Microbial Risk Assessment (QMRA) scheme (CAC, 1999) can provide insight on the impact of this step on the final risk of consumers, putting this process operation into a broader public health context (Posada-Izquierdo et al., 2014). QMRA outcomes can be later used to develop and assess risk metrics, such as Performance Objectives (PO) and Performance Criteria (PC) in relation to a specific Food Safety Objective (FSO) (Augustin and Guillier, 2018).

Therefore, this work aimed to i) study the effect of sodium hypochlorite in combination with BITC considering, in addition, the influence of disinfection process parameters (cut size, contact time and temperature) on the reduction of *Salmonella* spp. in fresh-cut vegetable during washing operations and ii) develop a suitable mathematical model to predict and optimize pathogen disinfection in fresh-cut vegetable products.

4.3 Materials and methods

Different methods were deployed to achieve the goals proposed in this work, covering microbiological aspects, experimental design, sample preparation, vegetable disinfection process simulation as well as statistical analysis and mathematical modelling. A general scheme is presented in Fig. 4.1, in which the experimental set-up and vegetable process steps are represented graphically.

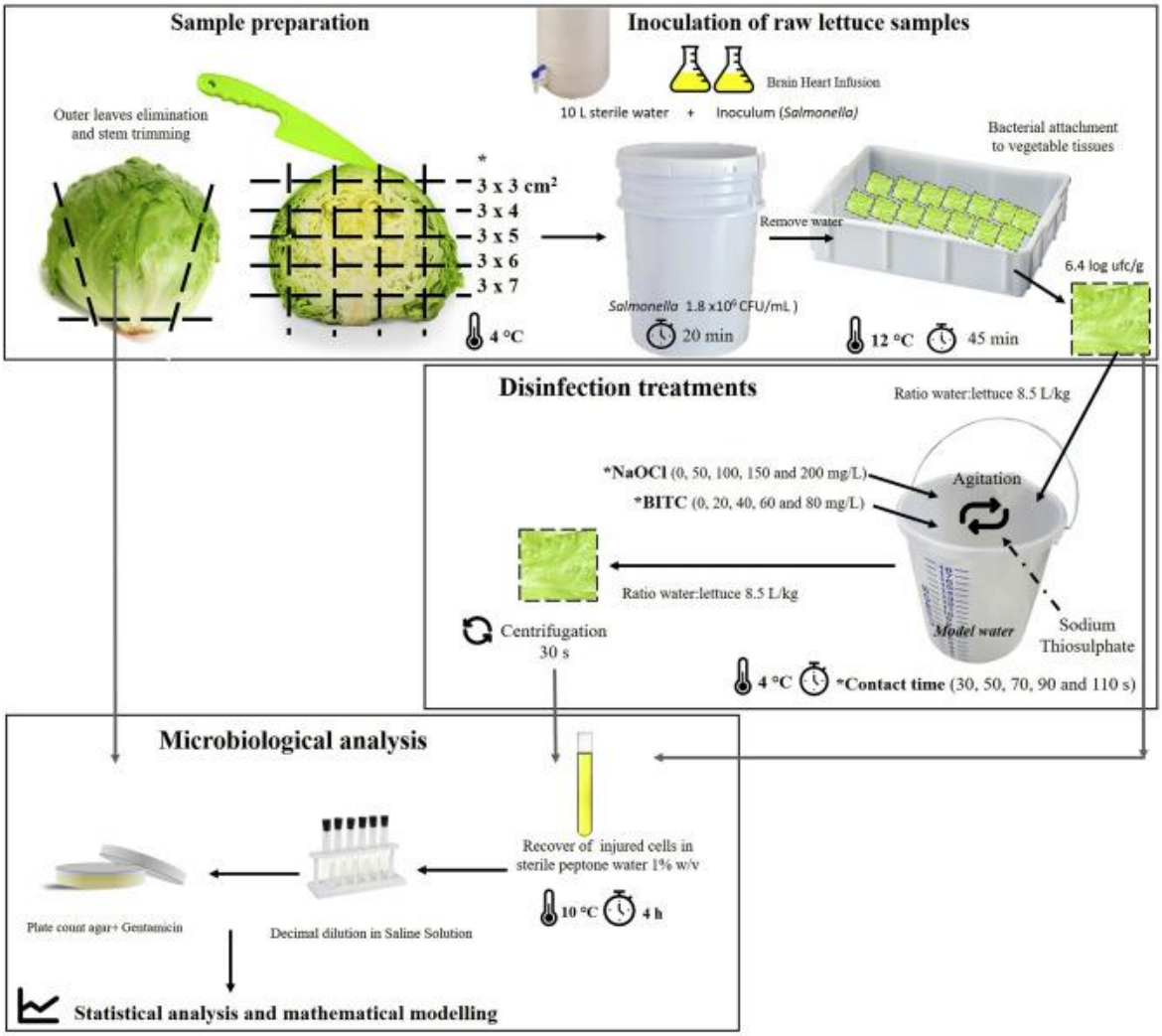

Figure 4.1. Graphic representation of experimental set-up and process steps

*Graphical representation of the experimental set-up comprising sample preparation, microbial inoculation and disinfection treatment of fresh-cut lettuce used to determine the effect of free chlorine, benzyl-isothiocyanate (BITC) concentrations (mg/L), lettuce cut size and contact time (denoted in graph with *) on Salmonella reductions (log CFU/g) on fresh-cut lettuce.*

4.3.1 Experimental design

A central composite design (CCD) (factorial design + star design for α value for rotability = 2) was implemented to study the effect on *Salmonella* reduction on lettuce; the factors evaluated were: lettuce cut size, washed time, and two disinfectants, BITC and sodium hypochlorite, at different concentrations. The five levels of the four factors are shown in Table 4.1. A total of 26 experiments were randomly performed in order to exclude the disturbing effects of environmental conditions (Robinson, 2000). All microbiological results

were obtained by duplicate determinations and in addition, the central point of the experimental design was duplicated (trials 6 and 17) in order to evaluate experimental error and thus lack-of-fit of the obtained model.

Table 4.1. Central composite experimental design for studying the effect of the selected variables on the reduction of *Salmonella* on lettuce.

Factors	Symbols		Levels				
	Uncoded	Coded	−α	−1	0	1	+α
Lettuce cut size (cm^2)	s	X_1	9	12	15	18	21
Contact time (s)	t	X_2	30	50	70	90	110
Benzyl-isothiocyanate (mg/L)	BITC	X_3	0	20	40	60	80
Sodium hypochlorite (mg/L)	NaClO	X_4	0	50	100	150	200

4.3.2 Sample preparation

Unprocessed heads of iceberg lettuce (Lactuca sativa L.) were obtained from a local market in Córdoba (Spain). After its reception in the laboratory, lettuce was stored at 4 °C and 60% relative humidity (RH) and used within 2 h upon the arrival in the laboratory in order to avoid any additional microbial and sensory deterioration. Lettuce heads were selected according to similar shape and size (450 g approx.).

Outer leaves and core were manually removed and discarded, while internal leaves were weighed to obtain 1.5 kg lettuce and then were cut into pieces of different sizes, in a clean area using plastic knives disinfected with 70% ethanol (Sigma-Aldrich, USA). The cutting process consisted of first cutting lettuce into halves, and then, each half was cut lengthways and transversely to produce lettuce pieces of approximately 3 × 3, 3 × 4, 3 × 5, 3 × 6 and 3 × 7 cm. All the processing steps were performed in a refrigerated room (6–8 °C) in order to maintain SWW at ca. 6 °C, which corresponds to the criterion followed by the vegetable industry.

4.3.3 Bacterial strain and inoculum preparation

A bacterial culture of Chromosomally Green Fluorescent Protein (GFP) labeled *Salmonella enterica* sv Thompson pGT-Kan mB156 was used in the study. The strain was prepared in cryobeads and kept in vials in a freezer at −80 °C. This strain was made resistant to gentamicin (15 μg/mL) to facilitate enumeration of *Salmonella* on fresh-cut iceberg lettuce, minimizing interferences from native microbiota.

For each independent experiment, the stock culture was re-activated by introducing a cryobead into 9 mL of Brain Heart Infusion (BHI, Oxoid, UK) broth, which was incubated at 37 °C for 24 h. Then, 1 mL of this suspension was added to 9 mL BHI broth and incubated at 37 °C for 24 h. Finally, two aliquots of 2.5 mL of this suspension were added to two flasks containing 22.5 mL BHI broth and incubated at 37 °C for 16 h to obtain inoculum to be used in the experiment.

4.3.4 Inoculation of raw lettuce samples

The inoculation process was carried out on 5 lots of 1.5 kg lettuce pieces each one. Lettuce pieces were immersed for 20 min in 10 L cold (4 °C) distilled and sterilized water with a *Salmonella* concentration of 5×10^6 CFU/mL, approximately. This concentration was obtained by inoculating the water with 50 mL pathogen suspension containing 8×10^8 CFU/mL *Salmonella*. Afterwards, each sample were taken out and placed on sterilized trays and maintained at 12 °C and 60% RH for 45 min in order to dry lettuce pieces while enabling bacterial attachment to vegetable tissues (Posada-Izquierdo et al., 2013). A 25 g-sample of the inoculated lettuce was analyzed after 45 min to determine the initial *Salmonella* concentration before disinfection experiments (Section 2.6). The initial concentration of *Salmonella* on the lettuce was 6.5 log CFU/g.

4.3.5 Disinfection treatment

4.3.5.1 Disinfection equipment

The disinfection treatment, which consisted of washing produces with chlorinated water, was performed at laboratory scale simulating process conditions of a real fresh-cut lettuce processing line from a Spanish processor. Polypropylene tanks with 5 L capacity were used to simulate the disinfection process, where the ratio water: lettuce corresponded to 8.5 L/kg as indicated by the Spanish processor.

4.3.5.2 Industrial process water

The water used in the disinfection process was prepared at laboratory following a procedure, which briefly consisted of sterilizing distilled water in autoclave (121 °C, 15 min) and then adding different chemical agents, under aseptic conditions, to reproduce the same physico-

chemical parameter values as those measured in a Spanish vegetable processing plant as reported by Pablos et al. (2018).

A summary of the chemical reagents and their concentration used for simulating process water and the main chemical properties are presented in Table 4.2. The total organic carbon (TOC) (150–150.3 mg/L) was obtained by adding 350 mg/L malt extract for microbiology (PanReac, Germany) and turbidity (100.1 NTU) was reproduced with 100 mg/L kaolin powder (Sigma Aldrich, USA) dissolved 24 h prior to use. Although this formulation does not represent the exact chemical composition of wash water, it has been proved to simulate adequately the effect of the organic matter and suspended solids present in real industrial samples. The pH (6.2–6.6) was modified by adding a 2 M NaOH solution. The resultant redox potential and conductivity corresponded to 500–543 mV and 1000–1096 μS/cm, respectively.

Table 4.2. Chemical properties and chemical agents used for the simulated the model water used in experiments.

Chemical properties		**Chemical agent**
Ca2+ (mg/L)	47.09	Calcium Chloride [c]
K+ (mg/L)	107.96	Potassium Chloride pharma grade [a]
Mg2+ (mg/L)	9.55	Magnesium Sulfate [c]
F^- (mg/L)	0.14	Sodium Fluoride 99% [a]
Cl^- (mg/L)	282.46	Sodium Chloride for analysis [b]
Br^- (mg/L)	10.21	Sodium Bromide pure pharma grade [a]
NO_3^- (mg/L)	51.57	Sodium Nitrate for analysis [a]
NO_2^-(mg/L)	0.03	Malt extract for microbiology [d]
SO_4^{2-} (mg/L)	51.02	Sodium Sulfate anhydrous for analysis [a]
NH_4^- (mg/L)	1.24	Ammonium Chlorine [c]
PO_4^{3-}(mg/L)	0.731	Malt extract for microbiology [d]
Na+ (mg/L)	87.73	Sodium Chloride for analysis [b]

[a] (Sigma Aldrich, Germany); [b] (Merck, Germany); [c] (PanReac, Spain); [d] (PanReac, Germany).

4.3.5.3 Disinfectant preparation

Prior to the disinfection process, 3.5 L simulated water was added in washing tanks and adjusted to the disinfectant levels. For that, a commercial solution of sodium hypochlorite at 10% (Sigma–Aldrich, USA) was added, in the washing tanks, at different volumes (17.5, 35, 52.5 and 70 mL) to obtain a free chlorine concentration of 50, 100, 150 and 200 parts per million (mg/L). These concentration values were confirmed by measuring free chlorine in simulated wash water (SWW) at time 0 of the disinfection process. These concentrations are similar within the range applied by the industry for washing fresh-cut vegetables in Spain and Argentina.

For BITC, a stock solution with a final concentration of 10,000 mg/L was prepared in ethanol using benzyl-isothiocyanate 98% purity (Sigma-Aldrich, USA). The BITC stock solution was added, in the washing tanks, at different volumes (7, 14, 21 and 28 mL) to get final concentrations of 20, 40, 60 and 80 mg/L. A control water tank was also prepared containing 0 mg/L of BITC and free chlorine following the conditions set by the experimental design.

4.3.5.4 Disinfection process

The disinfection process was applied to 410 g of inoculated fresh-cut lettuce pieces, by incorporating them in a washing tank containing SWW with the disinfection agent(s). Samples were washed under agitation with different contact times (30, 50, 70, 90 and 110 s). The disinfection process (i.e. oxidizing effect) was halted at each contact time by adding 30 mL Sodium Thiosulphate 0.05 M (Sigma-Aldrich, USA), as neutralizer, into the washing tank (López-Gálvez et al., 2009). The sample was collected from each tank by using a sterilized plastic strainer and centrifuged for 30 s using a manually operated centrifuge (5 L) (INTEY-EU), to remove the excess of water.

4.3.5.5 Physico-chemical parameters

The pH, oxidation/reduction potential (mV), total dissolved solids TDS (mg/L), turbity (NTU), conductivity (μS/cm) and temperature were measured before and after disinfection using the Multi-Parameter PCSTestr 3.5 (Oakton, USA). Free chlorine concentration was measured in the stock disinfectant solution and washing tanks before experiments by using the meter HI93734 (Hanna Instruments, UK).

4.3.6 Microbiological analysis

Processed lettuce samples (25g) were placed into Stomacher bags with 225 mL sterile peptone water 1% w/v (PW, Oxoid, UK). Then, stomacher bags containing lettuce samples were incubated for 4 h at 10 °C in order to recover *Salmonella* cells injured by the disinfection process (Liao and Fett, 2005). After the recovery step, samples were homogenized for 1 min using a stomacher (IUL Instruments, Spain). Decimal dilution series were prepared in tubes with 9 mL sterile saline solution (Merck, Germany) and plated into PCA (Plate Count Agar, Oxoid, UK) supplemented with gentamicin and incubated for 24 h at 37 °C. Characteristic *Salmonella* colonies exhibiting green fluorescence under an UV light were enumerated. The microbiological counts for *Salmonella* were expressed as log10 colony forming units (CFU) per gram (log CFU/g).

4.3.7 Statistical analysis and mathematical modelling

The reduction of *Salmonella*, produced by the disinfection treatment, was calculated as the difference, in decimal log scale, between initial (N0, log CFU/g) and final levels on lettuce (N, log CFU/g) (i.e. levels before and after treatment, respectively). This variable was used as response (i.e. dependent variable) in the statistical analysis. Response surface methodology (RSM) was the empirical procedure followed to study the relationship between the selected independent variables (lettuce cut size, time, BITC and free chlorine) and the dependent variable (i.e. reduction of *Salmonella* on lettuce, log CFU/g). Prior to the statistical analysis of the data, the values of the independent variables were normalized applying the codification according to Table 4.1, defined as dimensionless with mean zero and the same spread or standard deviation (Myers and Montgomery, 1995). In addition, a polynomial function was derived from the RSM, describing the effect of the studied variables on the reduction of *Salmonella*. The goodness-of-fit index adjusted R^2 (R2adj) was used to determine how well the model fitted to the experimental data. The statistical package Statistica® for Windows (version 11, Statsoft Inc., USA) was used for statistical analysis and mathematical modelling.

4.3.8 Model implementation and application in the software MicroHibro

The polynomial function derived from the RSM was deployed as a model for predicting disinfection of *Salmonella* on lettuce. To this end, it was implemented into MicroHibro

(www.microhibro.com), a predictive microbiology software toolbox with capabilities to perform quantitative microbial risk assessment within a stochastic environment (González et al., 2019). To determine the impact of disinfection on the fate of the microorganism along the food chain, subsequent potential growth steps (i.e. retail and household storage) were considered by integrating a suitable growth model available at MicroHibro (Sant'Ana et al., 2012). In this case, growth model variables were defined using available information and including variability, when appropriate. The model was simulated by using the Monte-Carlo analysis features of MicroHibro, and the variation in the number of final *Salmonella* at the moment of consumption were estimated.

4.4 Results and discussion

The initial concentration on inoculated lettuce before washing (N0) was 6.5 ± 0.2 log CFU/g. The inactivation data expressed as logarithmic reductions (N0–N) of viable cells achieved for each of the 26 experiments conducted in accordance with CCD are summarized in Table 4.3. Overall, the combinations of disinfectants and process parameters resulted in a mean reduction of *Salmonella* of 2.5 log CFU/g and a maximum (Nmax) and minimum reduction (Nmin) of 3.1 and 1.8 log CFU/g, respectively. Similar values were reported by other studies when disinfectant solutions of 100–200 mg/L free chlorine were applied (Osaili et al., 2018; Stopforth et al., 2008; Van Haute et al., 2013). In our study, the minimum reduction was obtained when no chlorine was used, being slightly higher than those values found by other works, that reported reductions of *Salmonella* between 0.5 and 1.2 log CFU/g for leafy vegetables washed with only water (Banach et al., 2017; Huang and Chen, 2018; Neal et al., 2012). The discrepancies between studies might be consequence of differences in the experimental setup (i.e. disinfection process parameters), including the addition of BITC as antimicrobial (Table 4.3).

Table 4.3. Results of *Salmonella* reductions after the disinfection process for the combinations of factors considered in the central composite design.

Trial	lettuce cut size (cm^2)	Contact time (s)	BITC (mg/L)	Sodium hypochlorite (mg/L)	*Salmonella* reduction (Log CFU/g)[a]
1	12	90	20	50	2.65±0.11
2	18	50	60	50	2.39±0.02
3	9	70	40	100	2.40±0.07
4	15	30	40	100	2.63±0.11
5	15	70	40	0	1.85±0.37
6	15	70	40	100	3.05±0.16
7	12	50	20	50	2.51±0.18
8	12	50	60	150	2.50±0.23
9	18	50	20	50	2.06±0.13
10	18	90	20	50	2.68±0.00
11	18	90	60	150	2.93±0.04
12	12	90	60	50	2.30±0.06
13	12	50	20	150	2.51±0.16
14	18	90	20	150	3.05±0.14
15	15	70	0	100	2.39±0.02
16	15	70	80	100	2.50±0.04
17	15	70	40	100	2.82±.025
18	12	50	60	50	2.29±0.04
19	12	90	20	150	2.81±0.01
20	12	90	60	150	2.85±0.11
21	18	50	20	150	2.58±0.08
22	18	90	60	50	2.14±0.04
23	21	70	40	100	1.97±0.28
24	15	70	40	200	3.00±0.21
25	15	110	40	100	2.95±0.11
26	18	50	60	150	2.42±0.01

BITC: Benzyl-isothiocyanate.
[a]Mean of reduction *Salmonella* of two replicates± standard deviation.

4.4.1 Effect of the disinfectants and process parameters on *Salmonella* reduction

The reduction of 1.8 log CFU/g (Nmin) observed when no disinfectant was used could be mostly related to the effect of the mechanical forces produced by sample agitation. This value was equal or even slightly higher than the magnitude of the variation range obtained for disinfection conditions (Nmax-Nmin = 1.3 log CFU/g). This fact highlights the great impact, in our study, of the mechanical release of cells from the vegetable tissue on *Salmonella* reductions observed in lettuce. Despite the variation range was not so wide, significant effects

could be seen for different factors, due to the well-controlled experimental environment, that reduced experimental variability (Table 4.3). According to this statistical analysis, free chlorine and BITC concentrations, contact time and cut size exerted a significant effect on the *Salmonella* reduction ($p \leq 0.05$). The statistical significance for the individual and combined effect of variables under study are presented in Table 4.4.

Table 4.4. Statistical analysis of the effect of the variables: free chlorine (mg/L), lettuce cut size (cmb), contact time (s) and benzyl-isothiocyanate (BITC) (mg/L) on *Salmonella* reductions on contaminated lettuce.

Variables	**Effect**
Lettuce cut size	-0.086
Lettuce cut size [b]	-0.348[a]
Time	0.233[a]
Time[b]	-0.046
BITC	-0.068
BITC[b]	-0.218[a]
Chlorine	0.411[a]
Chlorine[b]	-0.228[a]
Surface x Time	0.069
Surface x BITC	0.006
Surface x Chlorine	0.099
Time x BITC	-0.114
Time x Chlorine	0.139
BITC x Chlorine	0.066

[a]Statistical significance ($p \leq 0.05$).
[b]Indicates quadratic effect.

Chlorine showed a significant positive linear effect ($p \leq 0.05$) and in less extent, a negative quadratic effect ($p \leq 0.05$), confirming chlorine as an effective disinfectant for reducing the *Salmonella* population in leafy vegetables as widely reported by a number of studies (Banach et al., 2017; Meireles et al., 2016; Pezzuto et al., 2016; Van Haute et al., 2013; Weng et al., 2016). Fig. 4.2 depicts the positive linear effect of chlorine reflecting that as free chlorine level augmented, the *Salmonella* reduction increased. However, the negative quadratic effect for this variable also revealed that the maximum disinfectant ability was not produced at the maximum disinfectant concentration assayed (i.e. 200 mg/L), but it was observed in the range 100–150 mg/L free chlorine (Table 4.3).

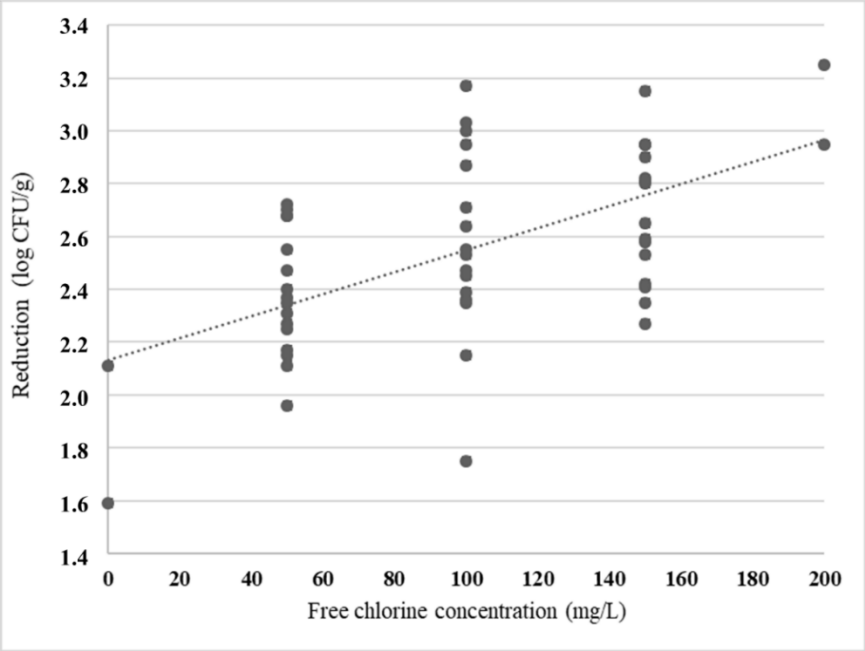

Figure 4.2. Linear effect of free chlorine concentrations (mg/L) on the reduction of *Salmonella* on fresh-cut lettuce (log CFU/g) (y = 2.1365 + 0.0041xChlorine) obtained from a central composite experimental design, including benzyl-isothiocyanate (0–80 mg/L), contact time (0-110 s) and the 26 experiments included in the experimental design.

Several studies have highlighted the importance of controlling pH during chlorine-based disinfection processes as a higher disinfection efficacy is achieved when chlorine is present in its undissociated form, as hypochlorous acid (HOCl), and chlorine demand of organic matter is reduced, which occur at low pH (i.e. acidic solutions) (Chen and Hung, 2017; Ramos et al., 2013; Waters and Hung, 2014). Indeed, the pH range recommended, in the vegetable industry, for effective and stable hypochlorite solutions is 6.5–7.5 (Chen and Hung, 2017; Suslow, 1997; Ward et al., 1984; Waters and Hung, 2014). In this study, no buffering or acids were used to control pH in washing water, therefore the addition of free chlorine resulted in changes of pH as shown in Fig. 4.3; in the same way, it can happen in some industries (i.e. the Argentine industry). This figure demonstrates that higher chlorine concentrations led to higher pH values, i.e. out of the recommended range. The pH increases up to values > 10 affected the chlorine efficacy, making that the highest reduction was not achieved at the maximum free chlorine level assayed (i.e. 200 mg/L). This phenomenon was captured by the statistical analysis through the negative quadratic effect found for free chlorine as mentioned above. A higher reactivity of chlorine to the organic matter from

lettuce (Chen and Hung, 2018; Waters and Hung, 2014) and a HOCl lower percentage (<30%), both aspects triggered by high pH values, could explain for the lower disinfection efficacy recorded at the highest chlorine levels (Davidson et al., 2013).

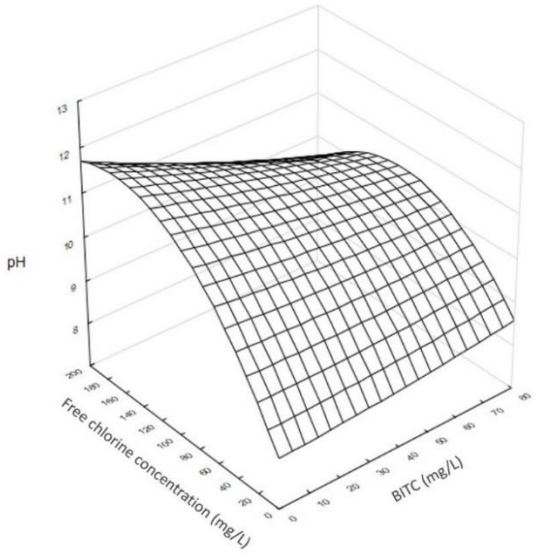

Figure 4.3. Response-surface plot showing the relationship between free chlorine and benzyl-isothiocyanate (BITC) concentrations (mg/L) and pH obtained from a central composite experimental design, including, in addition, the variables, contact time (30–110 s) and lettuce cut size (9–21 cm^2) fixed to 30 s and 9 cm^2, respectively. Circles represent the values observed from the experiments.

The formulation of BITC in the washing tank influenced pH, as evidenced by Fig. 4.3, in which increasing BITC concentrations yielded a slight reduction of pH, especially at higher chlorine values (>100 mg/L). However, the statistical analysis did not identify a significant interaction of chlorine and BITC.

The statistical analysis also showed a negative quadratic effect of BITC on *Salmonella* reduction, detonating that the optimum value did not coincide with the maximum level assayed (80 mg/L) in contrast to that expected and observed in previous studies (Pablos et al., 2018); therefore, the maximum efficacy in reducing *Salmonella* was reached at an intermediate value, which corresponded to 40 mg/L. Furthermore, it was observed that the addition of BITC at intermediate level (40 mg/L) increased the *Salmonella* reductions in 0.6 log CFU/g for a disinfection process with a free chlorine concentration in water of 100 mg/L, processing time of 60 s and lettuce piece size of 15 cm^2, in comparison with the experiments under the same conditions but performed without BITC. As evidenced by these data, the reduction levels associated with this compound were not particularly high despite the

statistical test detected a significant impact. Nevertheless, lower reductions were obtained, by other authors, for *Salmonella* on leafy green and fruits using a similar BITC concentration range (40–80 mg/L), even though no chlorine was added as disinfectant (Pablos et al., 2017; Romeo et al., 2018).

Despite no interaction with chlorine was detected as mentioned above, it cannot be discarded that the pH reduction observed in presence of BITC could enhance the disinfection ability of chlorine.

To determine the effect of contact time on the reduction of *Salmonella* during disinfection, washing was performed applying different process times, from 30 to 110 s. As expected, the increase in exposure time resulted in a significant greater reduction of *Salmonella* ($p \leq 0.05$) (Table 4.4) as reflected in Fig. 4.4. These results are consistent with those shown in other works, suggesting that the exposure time is a factor interacting with the efficacy of chlorine and BITC (Pablos et al., 2018; Tirpanalan et al., 2011). At the same factor combinations (free chlorine = 100 mg/L, contact surface = 15 cm^2 and BITC = 40 mg/L), the rise of contact time from 30 to 110 s increased *Salmonella* reduction in 0.7 log CFU/g. Although in our study, no shorter contact times were assessed, it is likely that changes in the range <30 s would yield a larger variation on *Salmonella* reductions, assuming that disinfection could mostly take place at the first seconds of the process, especially at high chlorine concentrations.

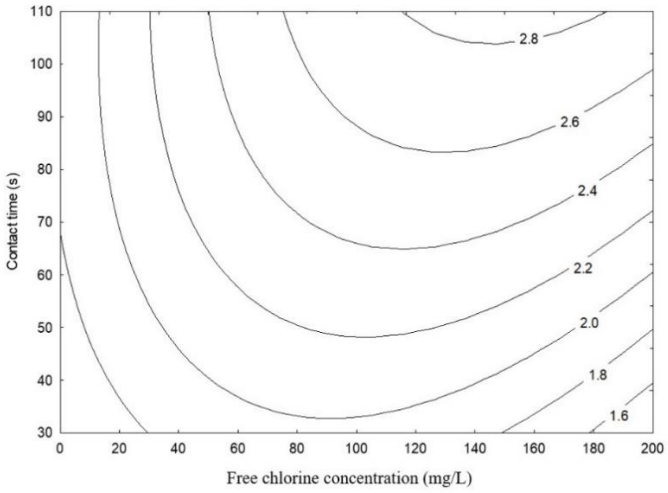

Figure 4.4. Contour plot showing lines of equal *Salmonella* reduction (log CFU/g) for the combination of free chlorine (mg/L) and contact time (s) obtained from a central composite experimental design, fixing lettuce cut size (cm^2) and benzyl-isothiocyanate (mg/L), to 12 cm^2 and 0 mg/L, respectively. Circles represent the values observed from the experiments.

As can be seen in Fig. 4.5, larger cut lettuce pieces yielded higher *Salmonella* reductions though its maximum effect was observed at 15 cm^2. Larger sizes than this value did not yield higher reductions.

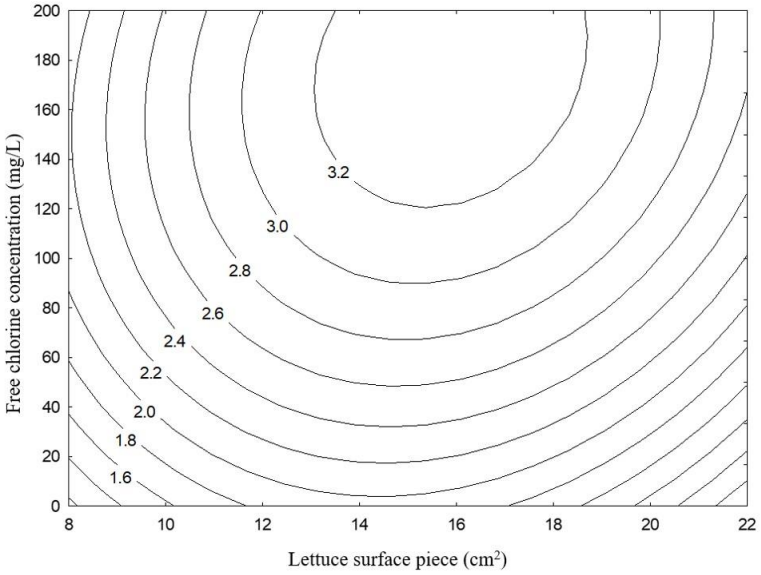

Figure 4.5. Contour plot showing lines of equal *Salmonella* reduction (log CFU/g) for the combinations of free chlorine (mg/L) and lettuce cut size (cm^2) obtained from a central composite experimental design, fixing contact time (s) and benzyl-isothiocyanate (mg/L).

Smaller size increases the cut-edge and number of wounds on the lettuce leaf tissue produced during the cutting process. Then, the organic matter released from these wounds into the washing solution can deplete free chlorine, leading to lower efficacy of the disinfectant against microorganisms (Nou and Luo, 2010; Guo et al., 2017). A larger piece size would reduce cut-edge extension and number of wounds, thereby reducing the amount of organic matter released in the washing water. Furthermore, an increase in cut-edge and wounds can create microenvironment protecting bacterial cells against environmental stress and favor microbiological proliferation in subsequent steps (e.g. storage). It has been demonstrated that bacterial pathogens such as *Salmonella* and *E. coli* O157:H7 preferentially attach to these cut edges and wounded tissues. Pathogen cells attached to wounded surfaces could infiltrate into the tissue and become protected from antimicrobial agents or processes (Annous et al., 2009; Li et al., 2008; Van der Linden et al., 2016).

The fact that values above 15 cm^2 resulted in worse performance of the disinfection process could not be explained based on results and variables monitored in the present work.

However, it can be hypothesized that lettuce cut sizes >15 cm^2 might reduce accessibility of disinfectant to vegetable surface for example by affecting agitation, thereby decreasing the microbial inactivation rate. A more specific study should be carried out to determine the cause(s) of the lower reduction observed at those cut sizes.

4.4.2 Optimum and industrial conditions for *Salmonella* disinfection

According to the CCD statistical analysis, the optimum combination of the different variables producing the highest reduction for *Salmonella* (3.2 log CFU/g) corresponded to a lettuce cut size of 15 cm^2 washed for 110 s in model water containing 160 mg/L free chlorine and 40 mg/L BITC. However, industry conditions can vary from the optimum combination obtained from the CCD developed in our study. For those cases, the polynomial expression described in Equation (1) resulting from the statistical approach can be deployed as model to predict the efficacy of a specific disinfection process, defined by a combination of values for the process parameters, provided those are within the model domain, i.e. variable ranges of the experimental design (Table 4.1).

Equation:

$$N_0 - N = -1.77758 + 0.00211 * Cl - 0.00005 * Cl^2 + 0.4908 * S - 0.01934 * S^2 + 0.00395 * t - 0.00006 * t^2 + 0.02598 * BITC - 0.00027 * BITC^2 + 0.00057 * S * t + 0.00005 * S * BITC + 0.00033 * S * Cl - 0.00014 * t * BITC + 0.00007 * t * Cl + 0.00003 * BITC * Cl \quad (1)$$

Where N0–N represents for the logarithmic reduction in *Salmonella* (log CFU/g); Cl is free chlorine concentration (mg/L), BITC is benzyl-isothiocyanate concentration (mg/L), t is the contact time (s) of disinfection process and S is the lettuce cut size (cm^2).

The F-value obtained for the model was 117.06, indicating that the model was significant ($p < 0.0001$). The coefficient of determination (R^2) and coefficient of determination adjusted (R2adj), which were equal to 0.84 and 0.82, respectively confirmed that the polynomial model was able to account for the observed *Salmonella* reduction (Fig. 4.6).

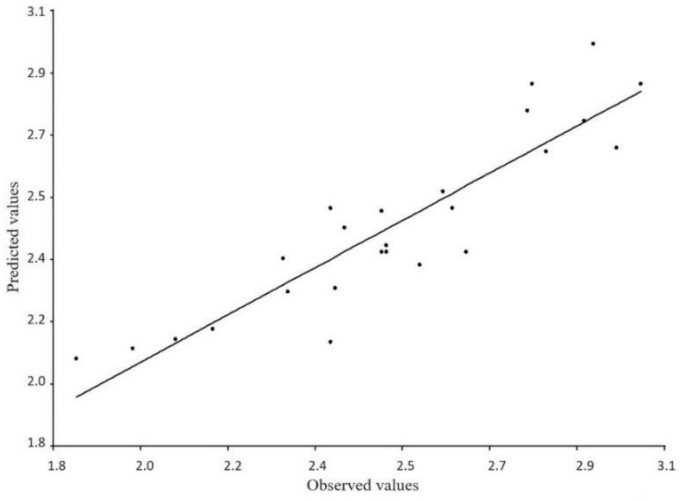

Figure 4.6. Predicted versus observed values for *Salmonella* reductions on fresh-cut lettuce. Prediction values were obtained from the polynomial function derived from a central composite experimental design.

Based on mathematical model predictions, potential control measures or process parameter modifications can be assessed. As an example, the effect of washing and disinfection conditions on a fresh-cut lettuce produced by a Spanish manufacturer was assessed using the mathematical model. The specific conditions for this industry were a process water temperature of 4 °C, a disinfection process time of 70 s and a commercial cut size of 12 cm^2. Under these conditions and according to the model predictions, the free chlorine concentration needed to achieve a *Salmonella* reduction of 2 log CFU/g was 50 ppm. The model also indicates that the disinfection process could be still equally effective (2 log reduction), if process time were reduced up to 50 s. On the contrary, when time is further reduced to 30 s, to maintain the same reduction, free chlorine concentration must be increased up to 100 mg/L. In the case of the Argentine industry where the disinfection process is typically developed with water at 4 °C for 60 s with 150 mg/L free chlorine and a commercial size for lettuce cuts of 16 cm^2; the model prediction returned a *Salmonella* reduction higher than 2 log CFU/g.

Although, to the best of our knowledge, hitherto, no industries use BITC as disinfectant for washing vegetables, according to the model, the use of this compound at small quantities (40 mg/L) in combination with chlorine (150 mg/mL) could increase *Salmonella* reduction in ~0.4 log with respect to reductions obtained without BITC. As mentioned above, it cannot

be discarded that this reduction is related to the low pH levels produced by BITC rather than a direct effect of this chemical compound. Regardless of the BITC action mechanism, this result could support the claim that combining different treatments can improve the effect of individual technologies since similar results can be obtained at lower concentrations of the individual disinfectants, reducing the negative impact on food quality while microbiological food safety is assured (Ge et al., 2013; Guo et al., 2017).

The European Food Safety Authority (EFSA) has established that the top ranking food/pathogen combination most often linked to foodborne human cases originated from Foods of Non-Animal Origin (FoNAO) in the EU is *Salmonella*s spp. and leafy greens eaten raw (EFSA, 2013; Silva et al., 2017). According to the Commission Regulation (EC) No 2073/2005 vegetables "ready-to-eat" must ensure absence of *Salmonella* in 25 g. The same requirement is included in the Article 925 of the Food Code (2017) in Argentina. Thus, in the hypothetical case of a batch of lettuce contaminated with *Salmonella* at 1 log CFU/g, which is a plausible value when *Salmonella* is present in leafy green vegetables according to results from several field surveys (EFSA, 2017; Herman et al., 2015; Sant'ana et al., 2011), the disinfection parameter combinations at the lowest values thereof ensuring absence of the pathogen in 25 g (−1.4 log CFU/g) corresponded to 50 s, 100 mg/L free chlorine and 20 mg/L BITC, considering a lettuce cut size of 12 cm^2.

Although washing and disinfection process with chlorine and BITC can reduce *Salmonella* levels, further studies are required to evaluate and model the behaviour of this pathogen during lettuce shelf-life, taking also into consideration the potential of resuscitation of injured cells.

As described above, most fresh produces are eaten raw or after minimal processing without any lethal treatment, enabling that pathogens can be present on produce at the moment consumption. Thus, the model developed in this study, along with supporting external validation data obtained on fresh vegetables can represent an important tool to establish appropriate processing criteria and the effective application of Sodium hypochlorite and BITC in combination with process parameters (lettuce cut size, contact time and temperature) on the reduction of *Salmonella* spp. on fresh-cut vegetable during washing operations.

4.4.3 Model application in a quantitative Microbiological Exposure Assessment

In this section, the disinfection model was applied into a quantitative Microbiological Exposure Assessment (MEA) scheme (WHO/FAO, 2008). This MEA study was aimed at determining the impact of a chlorine-based disinfection treatment on the exposure levels of *Salmonella* spp. in lettuce, when a contaminated lot enters the processing line. To this end, a probabilistic MEA model was built in MicroHibro, covering from industry to consumption by considering three main steps: i) Disinfection at factory, ii) distribution-retail cold chain and, iii) household storage. The variability associated with both the disinfection process parameters and the cold chain conditions was represented in the model through probability distributions. The model flow diagram as was built in MicroHibro is represented in Fig. 4.7. Input parameters used in the MEA model are presented in Table 4.5. Data from a Spanish ready-to-eat (RTE) vegetable company were collected for the disinfection treatment and the distribution-retail chain. In the latter case, measurements recorded by data loggers located in trucks and refrigerated display cabinets at retail from 50 different distribution routes (i.e. different destination across Spain) were used to estimate mean temperature and time. Household storage temperature and time were obtained from two previous studies developed in our laboratory, dealing with RTE vegetables specifically (Carrasco et al., 2010, 2007). *Salmonella* levels are usually very low in vegetables and when present, only presence/absence data are reported. Therefore, due to the lack of information, the initial concentration of *Salmonella* on unprocessed lettuce coming into the factory was assumed to follow a uniform distribution with the minimum being −2.4 log CFU/g (i.e. one cell in a 250-g lettuce bag) and the maximum corresponding to 3 log CFU/g. The latter parameter value was based on a published survey reporting one organic lettuce sample contaminated by *Salmonella* Typhimurium with levels of 2.94 log CFU/g (Sant'ana et al., 2011). The disinfection process was estimated using the polynomial model developed in the present work whereas the growth potential during the distribution-consumption chain was described by the growth model developed by Sant'Ana et al. (2012) for *Salmonella* on ready to eat lettuce packaged under modified atmosphere. The MEA model was simulated by using numerical techniques (i.e. Monte-Carlo analysis), providing output for intermediate steps and the level of consumption, in terms of concentration and prevalence of the pathogen. According to the simulated results, the disinfection process was able to remove 40% prevalent lettuce samples and reduce mean concentration by 0.9 log CFU/g (from 0.2 to −0.7

log CFU/g) while maximum levels dropped from 3 to 0 log CFU/g, approximately. Along the cold chain, *Salmonella* was able to rise reaching a mean level at the moment of consumption of 0 log CFU/g (i.e. one cell per gram) and a maximum value of 5.7 log CFU/g. Results demonstrated that the disinfection treatment was not completely effective when a heavily contaminated lot entered the processing line suggesting that variability in the initial contamination, disinfection treatment and storage conditions could increase the chance to result in more risky combinations of factors thus enabling the pathogen to reach consumers, even at high levels. For further detail, the exposure model simulation can be directly accessed on MicroHibro at http://www.microhibro.com/risks/

Table 4.5. Inputs used for the probabilistic exposure assessment model built in the quantitative risk assessment software, MicroHibro (González et al., 2019).

Step	Parameter		Units	Values/Distributions [a]	Source
	[Cl]	free chlorine	ppm	Uniform (20, 60)	RTE vegetable company
Disinfection	t_w	washing time	s	Uniform (60, 70)	RTE vegetable company
	C_z	Cut size	cm^2	12	RTE vegetable company
Distribution-retail	T_{d-r}	temperature	°C	Normal (6.35, 2.83; -1.1, 20)	RTE vegetable company
	t_{d-r}	time	hours	Exponential (29, 2, 298)	RTE vegetable company
Household	T_h	Temperature	°C	Normal (6.78, 2.56, 1, 11.3)	Carrasco et al. (2007)
	t_h	time	hours	Triangular (12, 84, 240)	Carrasco et al. (2010)

a) The distribution parameters corresponded to: Uniform (minimum, maximum); Normal (mean, standard deviation, minimum, maximum); Exponential (mean, minimum; maximum); Triangular (minimum, most probable, maximum).

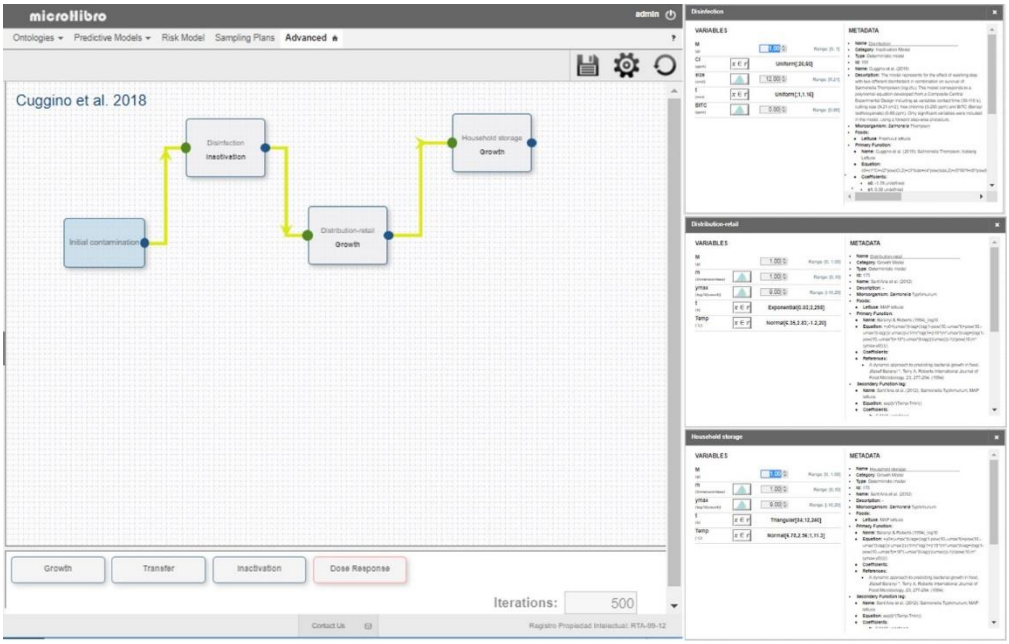

Figure 4.7. Snapshot of the probabilistic Microbiological Exposure Assessment model for *Salmonella* spp. on fresh-cut lettuce developed in MicroHibro (www.microhibro.com), including a chlorine-based disinfection treatment, represented by the polynomial function derived from a central composite experimental design.

4.5 Conclusions

Results, in the present study, confirmed the well-known efficacy of chlorine to reduce *Salmonella* populations in fresh-cut lettuce, during the washing step, while highlighting the importance of controlling the washing process parameters, such as contact time and lettuce piece size, in order to increase disinfectant efficacy and improve food safety. The work outcomes also showed that the use of the natural antimicrobial benzyl-isothiocyanate in combination with chlorine could slightly improve the disinfection performance, even though this effect could be related to a pH reduction rather than a direct action of benzyl-isothiocyanate. The polynomial model generated in the present study was suitable to predict *Salmonella* disinfection on lettuce under specific processing conditions, thus providing scientific support in the decision-making process. As illustrated in this work, the mathematical model could be also useful to develop studies of quantitative MEA, assessing the impact of the washing step and process parameters on the risk of this enteropathogenic bacterium in leafy green products. The output of the MEA study denotes disinfection as a crucial step to eliminate the pathogen, able to wipe out the highly contaminated produces,

though it was unable to fully remove *Salmonella* in the contaminated food batch enabling that the pathogen may grow during the cold chain and reaches final consumers.

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CAPÍTULO 5

5. Capítulo 5: Efectos de los tratamientos de lavado con cloro y ácido peroxiacético sobre la cinética de crecimiento de Salmonella en lechugas precortadas

Este trabajo se encuentra aceptado para su publicación en la revista Food Research International, con el título: "*Effects of chlorine and peroxyacetic acid wash treatments on growth kinetics of Salmonella in fresh-cut lettuce*". Sofia Griselda Cuggino, Isabel Bascón Villegas, Guiomar Posada-Izquierdo, Martin Gustavo Theumer, Fernando Pérez-Rodríguez. Fecha de aceptación 31 de diciembre de 2022.

5.1 Resumen

Los vegetales frescos cortados a menudo se consumen crudos, por lo que la desinfección adecuada es esencial para prevenir brotes de enfermedades transmitidas por los alimentos. Se evaluó la reducción y posterior crecimiento de *Salmonella* enterica sv Thompson en lechuga iceberg pre-cortada lavada con agua simulada modelo, hipoclorito de sodio (cloro libre 25 mg/L) y ácido peroxiacético (PAA, 80 mg/L) y almacenado durante 9 días en atmósfera modificada a 9, 13 y 18 ºC. Las diferencias en la reducción entre SH y PAA fueron inexistentes. En general, la calidad visual, la deshidratación, el pardeamiento en los bordes y en la superficie de las hojas, y el aroma durante el almacenamiento a 9 ºC, fueron similares entre los tratamientos, pero los efectos negativos aumentaron con la temperatura. Estos resultados demuestran que el peroxiacético puede ser utilizado como una alternativa eficaz al cloro para la desinfección de *Salmonella* spp. en lechuga fresca cortada. El crecimiento de *Salmonella* enterica sv Thompson se describió con éxito con el modelo primario de crecimiento de Baranyi y Roberts en el rango de temperatura de almacenamiento estudiado y después del tratamiento con agua, cloro y PAA. Posteriormente, se utilizaron modelos secundarios predictivos para describir la relación entre las tasas de crecimiento y la temperatura basados en la familia de modelos descrita por Bělehrádek. Curiosamente, la exposición a desinfectantes sesgó la cinética de crecimiento de *Salmonella* spp. durante el almacenamiento Por debajo de 12 ºC, las tasas de crecimiento en lechuga tratada con desinfectante (0.010-0.011 log UFC/h 9 ºC) fueron menores que las de lechuga lavada con agua (0.016 log UFC/h 9 ºC); mientras que, a temperaturas más altas, el efecto fue el contrario. En este último caso, los valores de la tasa de crecimiento registrados a 18 ºC para la lechuga tratada con desinfectante fueron de 0,048-0,054 log UFC/h frente a un valor de

0,038 log UFC/h para la lechuga tratada solo con agua. Los datos y modelos desarrollados en este estudio serán cruciales para describir estas interacciones en un marco de evaluación de riesgos aplicado a productos frescos cortados, proporcionando estimaciones de riesgo más completas y precisas.

Effects of chlorine and peroxyacetic acid wash treatments on growth kinetics of *Salmonella* in fresh-cut lettuce

Abstract

Fresh-cut produces are often consumed uncooked, thus proper sanitation is essential for preventing cross contamination. The reduction and subsequent growth of *Salmonella enterica* sv Thompson were studied in pre-cut iceberg lettuce washed with simulated wash water (SWW), Sodium hypochlorite (SH, free chlorine 25 mg/L), and peroxyacetic acid (PAA, 80 mg/L) and stored for 9 days under modified atmosphere at 9, 13, and 18 ºC. Differences in reduction between SH and PAA were non-existent. Overall, visual quality, dehydration, leaf edge, and superficial browning and aroma during storage at 9 ºC were similar among treatments, but negative effects increased with temperature. These results demonstrated that PAA can be used as an effective alternative to chlorine for the disinfection of *Salmonella* spp. in fresh-cut lettuce. The growth of *Salmonella* enterica sv Thompson was successfully described with the Baranyi and Roberts growth model in the studied storage temperature range, and after treatment with SWW, chlorine, and PAA. Subsequently, predictive secondary models were used to describe the relationship between growth rates and temperature based on the models' family described by Bělehrádek. Interestingly, the exposure to disinfectants biased growth kinetics of *Salmonella* spp. during storage. Below 12 ºC, growth rates in lettuce treated with disinfectant (0.010-0.011 log CFU/h at 9 ºC) were lower than those in lettuce washed with water (0.016 log CFU/h at 9 ºC); whereas at higher temperatures, the effect was the opposite. In the latter case, the growth rate values registered at 18 ºC for lettuce treated with disinfectant were 0.048-0.054 log CFU/h compared to a value of 0.038 log CFU/h for lettuce treated with only water. The data and models developed in this study will be crucial to describing these interactions in a risk assessment framework applied to fresh-cut produce, providing more complete and accurate risk estimates.

Keywords: predictive models, growth rate, Bělehrádek model, lettuce disinfection, microbial risk assessment, Modified Atmosphere Packaging, cross contamination, internalization, bacterial attachment.

5.2 Introduction

Over the last decades, our society has changed its consumption patterns, leading to an increased demand for fresh, healthy, safe, and easy-to-prepare food products (del Carmen Rodríguez et al., 2017; Guo et al., 2017; Yousuf et al., 2020). Governments and organizations promote the consumption of fresh fruits and produce as a part of a healthy diet and to reduce the incidence of certain diet-related diseases (e.g., cardiovascular, obesity, etc.) (Department of Health and Human Services and U.S., 2020; EFSA, 2010). Therefore, fresh produce and minimally processed vegetables have gained popularity worldwide (Castro-Ibáñez et al., 2017; Mir et al., 2018).

The consumption of fresh and freshly cut produce has been linked to several foodborne disease outbreaks as a consequence of the presence of several pathogenic bacteria, such as *Listeria monocytogenes*, *Clostridium botulinum*, *Bacillus cereus*, *Escherichia coli* O157:H7, and *Salmonella* sp. (Balali et al., 2020; Callejón et al., 2015; Centers for Disease Control and Prevention, 2019; EFSA. European Food Safety Authority., 2017; Iwu & Okoh, 2019; Machado-Moreira et al., 2019).

The number of outbreaks associated with the consumption of contaminated fresh products has increased all over the world (Castro-Ibáñez et al., 2017; EFSA, 2013; Iwu & Okoh, 2019; Machado-Moreira et al., 2019). Studies carried out in South America have reported the presence of *Salmonella* sp. in minimally processed vegetables (Gentili et al., 2017; Gómez Albanys et al., 2018; Maistro et al., 2012; M. A. de Oliveira et al., 2011).

The growing trend might be related to an increase in the consumption of fresh produce, the contamination from livestock farming near crop areas, the rapid global availability of foodstuffs sometimes produced in areas with unknown hygienic conditions, and the increase of susceptible population groups, with a higher number of immunocompromised consumers (Beuchat, 2002; Olaimat & Holley, 2012).

Salmonella sp. can survive in soil and treated waters, as well as on fresh produce for long periods (i.e., in term of months), particularly at cold temperatures (EFSA. European Food Safety Authority., 2014; Jacobsen & Bech, 2012). Controlling *Salmonella* sp. contamination requires a systematic approach that includes several aspects from farm to table: the quality of the raw material, the efficacy of the sanitation steps to prevent cross-contamination throughout the production chain, and appropriate storage temperatures (Castro-Ibáñez et al., 2017; Mir et al., 2018; Semanda et al., 2018).

Several researchers have emphasized the importance of washing and sanitizing treatments (Gil, Selma, et al., 2015; López-Gálvez et al., 2019; Meireles et al., 2016). A proper application of disinfection treatments could reduce pathogens in washing water, preventing cross-contamination (Banach et al., 2015; López-Gálvez et al., 2020; Maffei et al., 2017).

Sodium Hypochlorite (SH) is the most widely used disinfectant in the vegetable industry (Sam Van Haute et al., 2013; Weng et al., 2016) due to its relatively low price, easy application, and wide spectrum of antimicrobial activity (Ramos et al., 2013). However, under certain conditions, this disinfectant has limited efficacy in reducing microbial loads because it is sequestered by organic matters and its action is highly pH-dependent (Chen & Hung, 2017; Cuggino et al., 2020; Waters & Hung, 2014; Weng et al., 2016).

In addition, it has been found that, during the washing process, harmful disinfection byproducts (DBPs) can be formed (López-Gálvez et al., 2019; T. Zhang et al., 2022). Besides having potential adverse consequences on environment and human health (Lee & Huang, 2019; Simpson & Mitch, 2021), these substances cause unpleasant flavor and odor in fresh foods, resulting in negative sensory responses (Gil, Gómez-López, et al., 2015; Gómez-López et al., 2014; Sam Van Haute et al., 2013).

Different approaches to reduce or replace the use of chlorine have already been developed, including biological methods, alternative chemical compounds, and physical technologies, as well as combinations thereof (Birmpa et al., 2013; Meireles et al., 2016; Pablos et al., 2018; Petri et al., 2015; S. Van Haute et al., 2015).

In this regard, peroxyacetic acid (PAA) has been proposed as a potential alternative to chlorine, as an antimicrobial capable of reducing microorganisms on fresh produce (Fallik, 2014; López-Gálvez et al., 2020; Osaili et al., 2018; P. Singh et al., 2018). PAA is less sensitive in the presence of organic matters that sodium hypochlorite and does not produce

harmful disinfection by-products (Lee & Huang, 2019; Lippman et al., 2020; Zoellner et al., 2018), thus resulting in a low environmental impact (Davidson et al., 2017; S. Van Haute et al., 2015).

It is important to highlight that processing fresh produce does not completely eliminate microbial contamination. This means that it is important to select correct packaging and controlling the time and temperature along the distribution and consumption chains to prevent growth of both pathogens and spoilage microorganisms (Castro-Ibáñez et al., 2017; de Frias et al., 2018; Luo et al., 2010).

Packaging plays a relevant role in the microbiological protection of fresh-cut products (Turatti, 2011; WHO/FAO (World Health Organization/Food and Agriculture Organization), 2008). It is very important the selection of the material, the conditions that the packaging generates and the relationship between the weight/volume of the packaging. Modified atmosphere packaging (MAP) has been introduced as an enhancement technology to extend the shelf life of RTE vegetables (M. Oliveira et al., 2010; Posada-Izquierdo et al., 2014; Ramos et al., 2013; Zhuang et al., 2014). Mainly, it provides a reduced partial pressure of O2 to retard browning and inhibits or delays the growth of spoilage microorganisms and foodborne pathogens (Horev et al., 2012; Jideani et al., 2017; Paillart et al., 2017).

The recommended temperature to ensure ready-to-eat vegetables quality and safety is 4ºC (FDA, 2008, 2012; Rediers et al., 2009), even though a high percentage of domestic and commercial refrigerators do not meet this temperature criterion (Andritsos et al., 2021; Atilio de Frias et al., 2015; Jofré et al., 2019; Jovanovic et al., 2022; Ovca et al., 2021; PJ et al., 2017; Tsironi et al., 2017).

Although numerous small-scale laboratory studies have been performed to assess the efficacy of disinfectants against pathogens on leafy greens (Beuchat et al., 2004; Y. Huang & Chen, 2011; Keskinen et al., 2009; Keskinen & Annous, 2011), little has been done on the influence of PAA and chlorine, as bactericidal agents, on the subsequent steps of the cold chain.

According to Ndraha et al., (2022) estimating the growth kinetics of microorganisms in a specific food type is very important, especially in the context of quantitative microbiological risk assessment (QMRA). Consequently, it is necessary to collect information on the growth kinetics of specific pathogens in fresh vegetables, considering different processing conditions and treatments.

The aim of this work was to quantitatively compare the disinfection efficacy of three sanitizing water treatments (water, SH, and PAA) against *Salmonella* sp. on cut iceberg lettuce and its subsequent growth potential at different storage temperatures (9, 13, and 18 °C), using a predictive modeling approach.

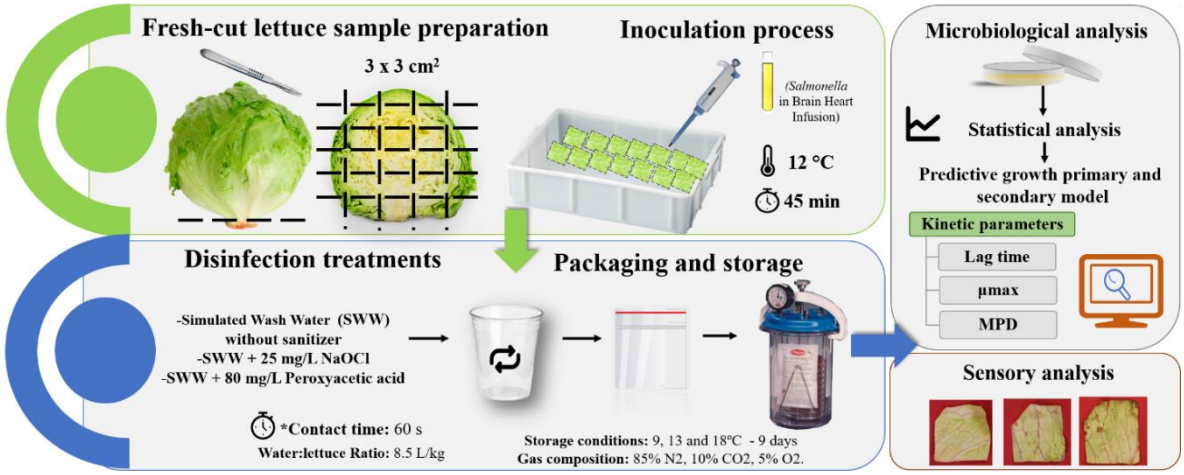


Figure 5.1. Graphic abstract: representation of experimental set-up and process steps.

5.3 Materials and methods

5.3.1 Fresh-cut lettuce sample preparation

Unprocessed heads of iceberg lettuce (Lactuca sativa L.) were obtained from a local market in Córdoba (Spain). The samples were selected, from the refrigerated shelves (4 ºC), according to their packaging date, which was usually the same as the harvest date. The transfer to the laboratory was carried out in coolers with ice, to keep the samples refrigerated. After its reception in the laboratory, lettuce was stored at 4 °C and used within 2 h upon arrival in the laboratory in order to avoid any additional microbial and sensory deterioration. The core and leaves of damaged lettuce were manually removed. The internal leaves were cut into commercial sized pieces of 3×3 cm (9 cm^2) under aseptic conditions. Sample processing was carried out in a refrigerated room (4 °C), simulating industrial conditions for the production of fresh-cut lettuce.

5.3.2 Bacterial strain, growth conditions and inoculate preparation

Salmonella enterica sv Thompson pGT-Kan mB156 gentamicin-resistant, labeled with green fluorescent protein (GFP), was used in this study to avoid potential interferences from

endogenous microbiota, allowing for a more precise and accurate enumeration of the inoculated pathogen. Cryoculture reactivation was carried out in Brain Heart Infusion (BHI, Oxoid, UK) and three consecutive subcultures were performed. Subsequently, cells from the last subculture were washed by centrifugation (Jouan C4i, Thermo Electron Corporation, France) at 4100 rpm for 10 minutes with a phosphate buffer (PBS, Medicago, Sweden). The pellet was resuspended in PBS and placed onto Plate Count Agar (PCA, Oxoid, UK) supplemented with gentamicin (15 µg/mL) and incubated at 37 °C for 24 hours to obtain the live culture. A colony of the live culture was streaked on PCA+ gentamicin plate, incubated at 37 °C for 16 h, and refrigerated (< 7 ºC) until experiments were conducted. The inoculum was prepared by adding the colonies into a saline solution (Panreac, Germany) up to approximately 2x107 CFU/mL. The concentration was initially adjusted using absorbance at 600 nm in a Bioscreen C analyzer (Labsystems, Helsinki, Finland) and then confirmed by plate count. Serial dilutions of the *Salmonella* inoculum were carried out to obtain the desired range of bacterial concentrations of ca., 5, 6, and 7 log CFU/mL.

The inoculation process was carried out on sterilized trays where each lettuce piece was individually inoculated with 0.1 mL *Salmonella* cell suspension in saline solution (0.85%, w/v) (Merck, Germany). The inoculum was spread on the lettuce piece homogeneously with a micropipette. The samples were then maintained at 12 ºC and 60% RH for 45 minutes to enable the drying of the inoculum suspension with bacteria attached to vegetable tissues.

The lettuce samples treated with disinfectant would result in lower *Salmonella* concentrations than those obtained in the experiments with only water, generating distinct starting microbial levels in the storage assay. As it could influence pathogen kinetics, in order to minimize its effect, the initial inoculum concentration for samples to be treated with only water was adjusted to one logarithm lower than that used in the experiments with disinfectant.

5.3.3 Disinfection treatments

5.3.3.1 Simulated industrial process water

Simulated wash water (SWW) was prepared based on the composition of water used in the Spanish vegetable processing industry following the procedure reported by Pablos et al., (2018) and Cuggino et al., (2020). Briefly, it consisted of formulating different chemical agents (ionic compounds, kaolin powder, malt extract, among others) in sterilized distilled water in order to reproduce the typical values of the main physicochemical parameters

registered in industrial process water. The values for pH (6.5), oxidation/reduction potential (530 mV), Total Organic Carbon (TOC 150 mg/L), turbidity (100 NTU (Nephelometric Turbidity Unit), conductivity /1050 µS/cm), total dissolved solids TDS (750 mg/L), and temperature were measured using the Multi-Parameter PCS Testr 3.5 (Oakton, USA) to monitor the established industrial parameters in order to verify that the model water processes were similar to those registered in the industry.

5.3.3.2 Simulation of the lettuce washing process

The inoculated lettuce pieces were immersed in a sterile plastic container with SWW. The water: lettuce ratio corresponded to 8.5 L/kg, being equivalent to the industrial ratio used for these products (Cuggino et al., 2020 and Pablos et al., 2018). Three types of treatments were tested, corresponding to SWW without sanitizer, and washing water formulated with SH or PAA.

For the disinfection treatments, the final concentration was 25 mg/L and 80 mg/L of free chlorine (Sodium hypochlorite, Sigma–Aldrich, USA) and PAA solution (Merck, Germany), respectively. The disinfectant levels were chosen on an industrial basis, and considering previous works, in which sensory impact was also evaluated as for the case of PAA (Beuchat et al., 2004; Code of Federal Regulations (CFR), 2012; Cuggino et al., 2020; Lippman et al., 2020; Osaili et al., 2018; P. Singh et al., 2018). The pH of the chlorinated water was adjusted to 6.5 with a solution of 0.1M HCl (Merck, Germany).

The free chlorine concentration and pH of the chlorinated water were monitored in the wash tanks using a HI93734 meter (Hanna Instruments, UK).

The samples were treated for 60 seconds under constant agitation at 4 ºC. The disinfection process (i.e., oxidizing effect) was halted at 60 s by adding 180 µL Sodium Thiosulphate (Sigma-Aldrich, USA), as a neutralizer, into the wash tank, avoiding that bacterial inactivation could extend beyond the time defined in the experimental set-up for washing (López-Gálvez et al., 2009; S. Van Haute et al., 2015). The samples were drained with a manual centrifuge (3.5 L) (model 23200 Leifheit, Germany) to remove excess water.

5.3.3.3 Packaging and storage conditions

The processed lettuce samples were aseptically packaged into individual sterile plastic bags, which were perforated, producing 4 holes Ø2 mm. The dimensions of the bags were 7×5×1

cm according to the weight/volume ratio used for commercial bags (250 g/1.38 L). Triplicates of packaged treated samples were placed in anaerobic jars (3.5 L) with the atmosphere generation sachet CampyGen (Thermo Scientific, Oxoid, Japan). As plastic bags were perforated, samples were exposed to the atmosphere generated in the jar, whose gas composition was: 85% N2, 10% CO_2, 5% O_2. These values are in line with the values registered in commercial packages for this type of vegetables. Packaged samples were stored at 9, 13, and 18 °C for 9 days. The temperatures are within the growth range of *Salmonella* and were selected because they represent potential scenarios of abuse of refrigeration temperature (9 °C and 13 ºC), and environmental temperature (18 °C) in households, supermarkets, and markets. The storage temperature was monitored with the MicroLite data logger (Fourier Technologies, Israel). The storage time was chosen based on the shelf life reported by manufacturers of these products. Samples were extracted for analysis on day 0 (the day experiments were performed) 3, 5, 7, and 9. At each sampling point, samples from each treatment were analyzed for sensory properties and *Salmonella* counts.

5.3.4 Microbiological analysis

The treated, untreated and packaged lettuce samples were microbiologically analyzed to enumerate *Salmonella*. For this purpose, lettuce pieces (~0.81 g) were placed into sterile tubes with 7.3 mL of 0.1% peptone water (PW, Oxoid, UK), and vigorously shaken by vortexing for 30 s (Vortex mixer, ZX3, VELP Scientifica Srl, Italia) under aseptic conditions. Homogenized samples were serially diluted in saline solution (0.85% NaCl) and plated in PCA supplemented with gentamicin to enumerate and identify the green fluorescence colonies of *Salmonella*. Microbiological counts of *Salmonella* were expressed as decimal logarithms of colony forming units (CFU) per gram (log CFU/mL). Each experiment was performed with three replicates of lettuce pieces and independently repeated three times.

5.3.5 Scanning Electron Microscopy Analysis

Lettuce leaf pieces were incubated with different concentrations of *Salmonella* (ca. 4-7 log UFC/mL) at 25 °C for 1 h, in the presence of light, following the inoculation procedure described above (sections 5.3.2 and 5.3.3). A subset of lettuce leaf pieces was then washed with water, simulating the washing process, following the same protocol as above (section 5.3.4). Internal 1×1 mm squares of treated lettuce samples were then excised under sterile

conditions and fixed at 7 °C for 24 h in 2% glutaraldehyde (Alfa Aesar, Germany). After that, samples were dehydrated in 30%, 50%, 70%, 90%, and 100% absolute acetone (Scharlau, Spain). They were then critical-point dried with CO2, mounted on a stand, and sputter-coated with a thin layer of gold in a high vacuum. Digital images were captured with a JOEL JSM 7800F scanning electron microscopy (SEM) (JOEL, Japan).

5.3.6 Imaging analysis and sensory assessment of lettuce samples

5.3.6.1 Image acquisition

To analyze the visual quality of the treated and stored lettuce, digital photographs were taken of samples during storage. The images were obtained using a digital camera (Canon EOS 1300D, USA). The camera was mounted on a stand adjusted to 30 cm above the base. To avoid capturing shadows and glare in the photographs, four fluorescent lights (27 W) were placed at different points, then the lettuce pieces were placed on a gray board and photographed. All experiments were conducted in a dark room at room temperature. The camera was set to focal length: 55.0mm on automatic indoor focus with the flash off. These settings provided a close-up view of the lettuce and covered the entire field of the sample. Three processed and packaged lettuce samples were photographed for each treatment and sampling time during storage at different temperatures. The captured pictures were exported to JPEG format for examination.

5.3.6.2 Sensory analysis

The sensory quality of the products after washing with and without different sanitizers was evaluated during storage by a sensory panel formed by 10 trained panelists, members of the Department of Bromatology and Food Technology of the UCO, and the Agri-Food Quality Transfer Center of the Faculty of Agricultural Sciences of the UNC. The sensory attributes evaluated corresponded to Overall visual quality (OVQ) (freshness and brightness), Dehydration (succulence and freshness), Leaf edge browning, and Leaf superficial browning, and Aroma (identified as off-odor) using a category test with modifications (Lopez-Galvez et al., 2013; Salgado et al., 2014; J. Zhang & Yang, 2017; Zhou et al., 2004). The sensory panel was trained to align with and adopt the descriptions for the sensory attributes proposed by Baur et al. (2004) and Oliveira et al. (2013). During training, the concept of each attribute was introduced through photos and lettuce samples that were selected according to the points

of the scale used. The above attributes were scored with a 9-point scale as shown in Table 5.1.

Attributes	Definitions	Sensory scores
Overall visual quality (OVQ)	Bright green color of fresh lettuce	1: absence of brightness/opaqueness/staling, 3: a little, 5: moderate, 7: a lot, 9: presence of brightness/shininess
Aroma	Absence of unpleasant or strange odor	1: severe off-odor, 3: strong off-odor, 5: moderate off-odor, 7: a little off-odor, 9: no off-odor
Leaf edge browning	Appearance of browning in edges	1: Severe browning (70-100% of edges browning), 3: moderately severe (50-70% of edges with browning), 5: moderate (50% of edges browning), 7: slight (less than 30% of edges browning), 9: No browning
Leaf superficial browning	Appearance of browning in the midrib and surface	1: Severe browning (70-100% of superficial browning), 3: moderately severe (50-70% of superficial with browning), 5: moderate (50% of superficial browning), 7: slight (less than 30% of superficial browning), 9: No browning
Dehydration	Loss of succulence and turgidity of leaf, indicative of freshness	1: Dry and flaccid, 3: severe dehydration and loss of succulence, 5: moderate dehydration, 7: mild dehydration and good succulence, 9: hydrated and succulent

Table 5.1. Definitions of the sensory attributes evaluated by the sensory panel and their corresponding sensory scores.

5.3.7 Growth model and kinetic parameter estimates

Salmonella counts were transformed into a decimal logarithmic scale and entered into Excel (Excel® 2010, Microsoft, Redmond/WA). The primary growth model of Baranyi & Roberts (1994) was fitted to growth data using the Excel DMFit 3.5 add-in (Institute of Food Research, Norwich, United Kingdom). Three kinetic parameters were estimated, namely, lag time (λ, expressed in h), maximum growth rate (μmax, expressed in log CFU/h), and maximum population density (MPD, expressed in log CFU/g). The MPD parameter represents the upper asymptote of the predicted growth curve; however, some curves did not reach this asymptote. In these cases, it was calculated as the maximum predicted concentration (Nmax, expressed in log CFU/g).

Secondary predictive models were also developed to represent the effect of temperature on μmax, based on the family of models described by Bělehrádek (1926) represented by Equation 1.

$$r = b \cdot (T - T_0)^m \qquad \text{Eq. 1}$$

In this model, r is a rate, b and T0 are regression parameters, and T is temperature in °C. This equation was later applied by Ratkowsky et al. (1982) and in other works (Ross, 1987, 1993) for microbial growth using m=2 and $r=\sqrt{\mu_{max}}$, with μmax being the maximum growth rate, and interpreting T0 as the notional minimum temperature for microbial growth, which is usually 5-10 ºC lower than the actual minimum temperature. This model is extensively used in predictive microbiology as a secondary model. Further, the works by Dantigny, (1998) and Dantigny & Molin (2000) reported that m values can range from 1 to 2, depending on whether the microorganism is mesophilic or psycrotrophic, respectively. In this work, Eq. 1 was fitted to data, using m= 1 and 2 and $r = \sqrt{\mu_{max}}$ and $ln(\mu_{max})$.The performance of the developed predictive models was evaluated using the Coefficient of Determination (R^2) and Standard Error (SE).

5.3.8 Data treatment and statistical analysis

The statistical analysis was performed using InfoStat version (2017) (Grupo InfoStat, Argentina). *Salmonella* reduction was calculated as the difference, on a logarithmic decimal scale, between bacterial counts in lettuce before treatment (N0, log CFU/g) and after treatment (Nf, log CFU/g). In addition, the survival and growth of *Salmonella* in cut, treated, and stored lettuce were studied. The growth potential (δ) of *Salmonella* was determined by the difference between the microbial counts at the end (day "9") (log10 CFU/g) and at the beginning (time "0") (log10 CFU/g) of shelf life (Beaufort, 2011). Three replicates per measurement were performed and data were expressed as the mean of the replicates. Differences between means were determined using Tukey's method with a confidence level of 95 % ($p< 0.05$).

5.4 Results and discussion

5.4.1 Effect of initial inoculum concentration on the reduction of *Salmonella* in fresh-cut lettuce during washing

The effect of initial inoculum concentration on *Salmonella* reduction in lettuce pieces after washing with SWW was studied using three different inoculum levels (ca. 4-6 log CFU/g). The positive correlation reported by the Spearman correlation coefficient (0.90, $p<0.0001$) indicated that *Salmonella* reduction increased significantly as the initial concentration of the inoculum increased. For graphical analysis, Figure 5.2 illustrates the initial inoculum concentrations vs. the reduction obtained at the end of the water treatment. The trend

exhibited by the data points evidences the positive association shown by the Spearman correlation coefficient. Moreover, a statistical comparison of the reductions at different inoculum levels confirmed that the reductions were significantly (p<0.05) higher (1.64 ± 0.32 CFU/g) in lettuce pieces contaminated with initial inoculum greater than 5.5 log CFU/g. In contrast, *Salmonella* inocula of 4.5-5.5 log CFU/g and lower than 4.5 log CFU/g resulted in lower reductions, corresponding to 1.04±0.12 and 0.96±0.04 log CFU/g, respectively, which were not statistically different (p>0.05).

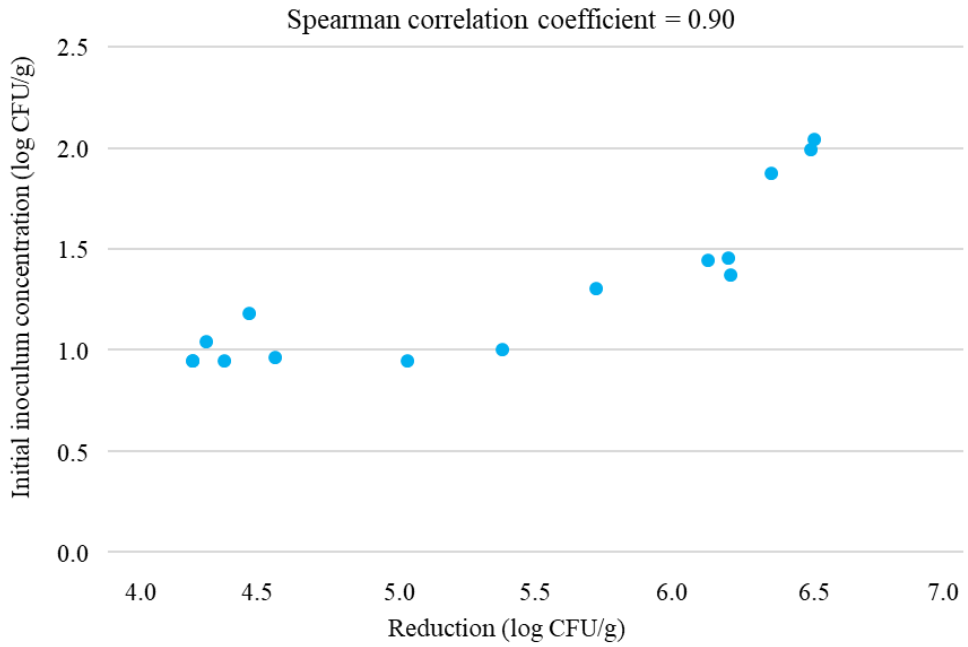


Figure 5.2. Initial inoculum concentration vs. reduction of *Salmonella* cells in fresh-cut lettuce (log CFU/g) at the end of the treatment with Simulated Wash Water (SWW) during 60 s.

The study by Van der Linden et al. (2016) observed that higher inoculum levels were associated with greater reductions of E. *coli* O157:H7 and *Salmonella* populations in the cut edges and the surface region of iceberg lettuce pieces. On the contrary, the leaf pieces inoculated with a low inoculum showed a smaller decrease in cells after washing, which was most clearly noted in the cut edges. This behavior was already reported for other pathogens such as E. *coli* O157:H7, which showed a greater attachment to cut edges of leaves, especially at lower inoculum levels (Takeuchi & Frank, 2001). In turn, large population densities cause cells to colonize sites other than wounds, stomata, cracks, and broken trichomes, presenting

a lower attachment capacity (Kazue Takeuchi & Frank, 2001) that could facilitate washing-induced removal.

In line with the above, SEM images obtained from the analyzed lettuce samples suggest that, at higher levels, *Salmonella* covered a larger surface area of leaf tissues, showing a greater presence of clustering compared to lower levels (Figure 5.3). In addition, the fact that experiments were performed under light conditions allowed stomata to be open, causing *Salmonella* to colonize near and inside the leaf stomata, as reported on other studies (Golberg et al., 2011; Kroupitski et al., 2009). The detachment of the most densely populated cell clusters on the flat and intact surface (cuticle) of leaf tissue could lead to a greater loss of *Salmonella* cells (Bermúdez-Aguirre & Barbosa-Cánovas, 2013; Takeuchi & Frank, 2001).

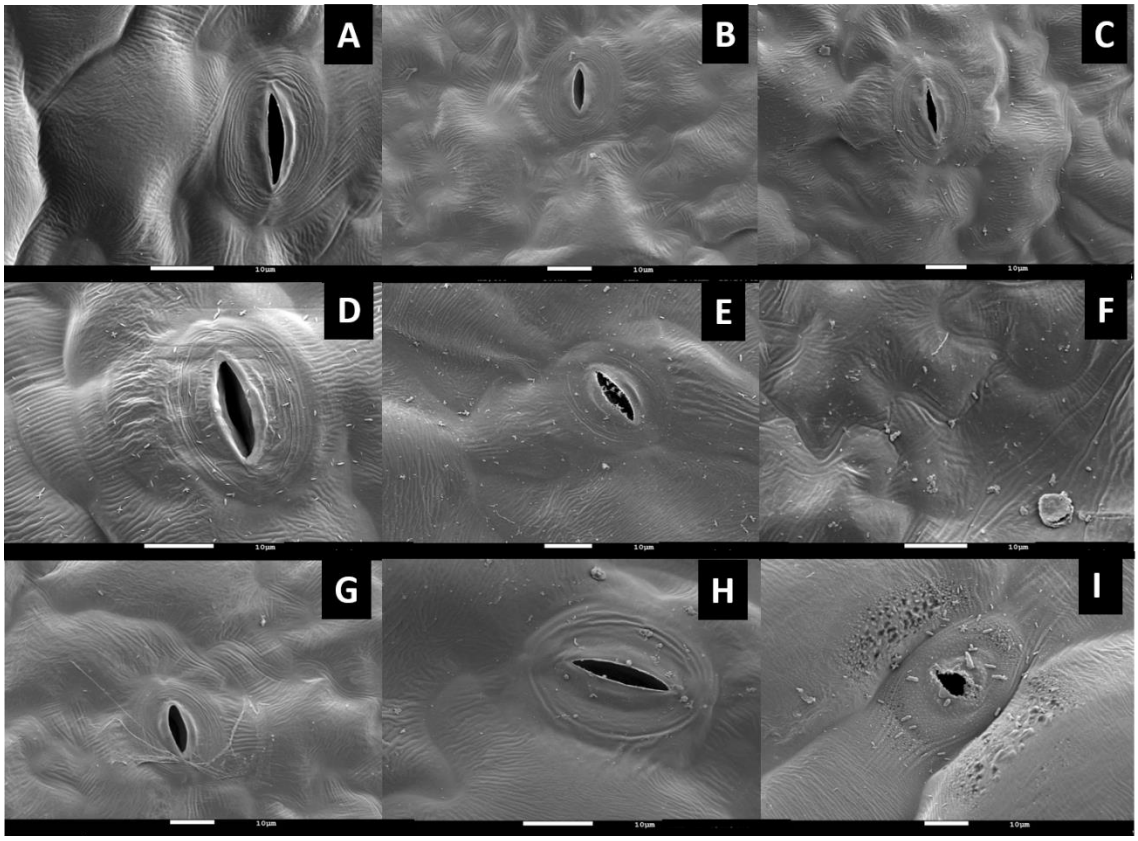


Figure 5.3.Scanning electron microscope (SEM) images of *Salmonella* cells on lettuce leaf surfaces of control samples (A), untreated samples inoculated at 4.4 log CFU/g (B), 5.5 log CFU/g (C), 5.3 log CFU/g (D), 6.5 log CFU/g (E), and 6.2 log CFU/g (F) and samples treated with simulated wash water (60 s) resulting in final levels of 3.5 log CFU/g (G), 4.5 log CFU/g (H), and 4.2 log CFU/g (I).

5.4.2 *Salmonella* reductions in fresh-cut lettuce washed with water and disinfectant solutions

The reductions on *Salmonella* populations in fresh-cut lettuce samples obtained with the different washing treatments are shown in Table 5.2. The initial inoculum size differed for each treatment in order to obtain similar final levels after treatment (i.e., the initial level for the storage phase). With this strategy, the possible influence of the initial concentrations on pathogen growth during the storage experiments was minimized (M. Coleman, 2003; López-Gálvez et al., 2018; Ma et al., 2016). Thus, lettuce samples treated only with SWW were inoculated with a lower inoculum, corresponding to 4.6 log UFC/g, and samples treated with disinfectants were inoculated with inoculum levels of around 6.2 log UFC/g (Table 2). Nevertheless, according to the results provided in the previous section, which demonstrate an influence of the initial inoculum on *Salmonella* reductions, the effect thereof was deemed less relevant compared to the reduction caused by the different disinfectants used.

Table 5.2. *Salmonella* reductions in fresh-cut lettuce washed with Simulated Wash Water (SWW), 25 mg/L chlorine (SH), and 80 mg/L peroxyacetic acid (PAA).

Treatment	N_0 (log CFU/g)	N_f (log CFU/g)	Reduction (log CFU/g)
SWW	4.61 ± 0.35	3.71 ± 0.40 [b]	0.91 ± 0.07 [b]
SH	6.28 ± 0.13	3.29 ± 0.10 [a]	2.98 ± 0.19 [a]
PAA	6.06 ± 0.45	3.27 ± 0.15 [a]	2.79 ± 0.36 [a]

Different letters in the same column indicate significant differences ($p<0.05$) according to Tukey.
N0: initial concentration of *Salmonella* in fresh-cut lettuce.
Nf: concentration of *Salmonella* in fresh-cut lettuce after washing with Simulated Wash Water (SWW), 25 mg/L chlorine (SH), and 80 mg/L peroxyacetic acid (PAA) during 60 s.

Salmonella populations were reduced by 0.91 ± 0.07 log UFC/g after washing cut lettuce samples with SWW for 60 s under agitation. The effect of wash water on microbial reductions reported by other studies using similar treatment times ranged from 0.4 to 1.6 log CFU/g (Cap et al., 2020; R. Huang & Chen, 2018; Li et al., 2017; Lippman et al., 2020; Neal et al., 2012; Pahariya et al., 2022). In contrast, the effect of incorporating 25 mg/L of SH and 80 mg/L PAA caused a decrease in *Salmonella* contamination by 2.98 ± 0.19 and 2.79 ± 0.36 log CFU/g, respectively (Table 5.2). These values were significantly higher than those obtained with water alone ($p<0.05$), but statistically similar between disinfectants ($p>0.05$).

The work by Huang et al. (2018) reported no significant differences between both treatments with PAA and SH. In line with our study, these authors observed that the use of these chemicals significantly increased the reduction of *Salmonella* in relation to the control treatment, composed only of SWW.

Similar reductions were reported in a previous work conducted in our laboratory (Cuggino et al., 2020) and by other authors (Osaili et al., 2018; Pezzuto et al., 2016; Stopforth et al., 2008; Sam Van Haute et al., 2013), but applying concentrations of disinfectant of 100-200 mg/L free chlorine. Pezzuto et al. (2016) found that washing raw rocket with 200 mg/L sodium hypochlorite reduced *Salmonella* counts by 2 logs.

For PAA, the results of our study indicated a greater *Salmonella* reduction compared to other studies (Lippman et al., 2020; Ruiz-Cruz et al., 2007). For instance, Ruiz-Cruz et al., (2007) showed that washing shredded carrots reduced *Salmonella* populations by 2.1 log CFU/g; however, the product and disinfection values used were different, i.e., 40 ppm PAA and 2 min agitation. In shredded iceberg lettuce, *Salmonella* was reduced by 1.52 log CFU/g when samples were exposed to 80 ppm PAA for 2 min (Lippman et al., 2020). In a similar study (Banach et al., 2020) applied to fresh-cut lettuce, E. *coli* reductions of 2.8-3.0 log CFU/g were obtained using 80 mg/L PAA, for 2 min at 4 ºC, equivalent to the values used in our study.

The differences in effectiveness found between studies, for both SH and PAA, are the result of several factors, namely food type, initial pathogen concentration, temperature, organic load, product to disinfectant solution ratio, type of washing treatment, pH of treatment solutions, concentration and disinfection time (Marçal et al., 2022; Pahariya et al., 2022).

5.4.3 Sensory quality during storage of fresh-cut lettuce washed with water and disinfectant solutions

The overall visual quality (OVQ) of the lettuce samples after processing (day 0) was scored by panelists at a mean of 7.91±1.19, 8.38±0.94, and 8.04±1.19 for products treated with SWW, SH, and PAA, respectively (Figure 3); indicating good brightness and freshness of lettuce samples, without significant differences between treatments. The relatively high standard deviation obtained in each group could be mostly due to the inherent variability of the processed lettuce leaf pieces, although the panelist assessment could be another relevant source of uncertainty in the scores. These results are in accordance with previous works, in

which similar OVQ scores were observed between washing treatments comparable to those evaluated in this study (Lopez-Galvez et al., 2013; Salgado et al., 2014).

All the attributes studied in the samples stored at 9 ºC were similar among treatments during storage (p>0.05). However, when the temperature increased, negative effects were observed on all attributes, which was more evident at 18 ºC. In this respect, other authors have reported that the combination of disinfectants and storage at higher temperatures increases the respiration rate of fresh-cut lettuce and water loss, resulting in a general deterioration of the lettuce pieces (Guan et al., 2010; Luna et al., 2013; Vandekinderen et al., 2009).

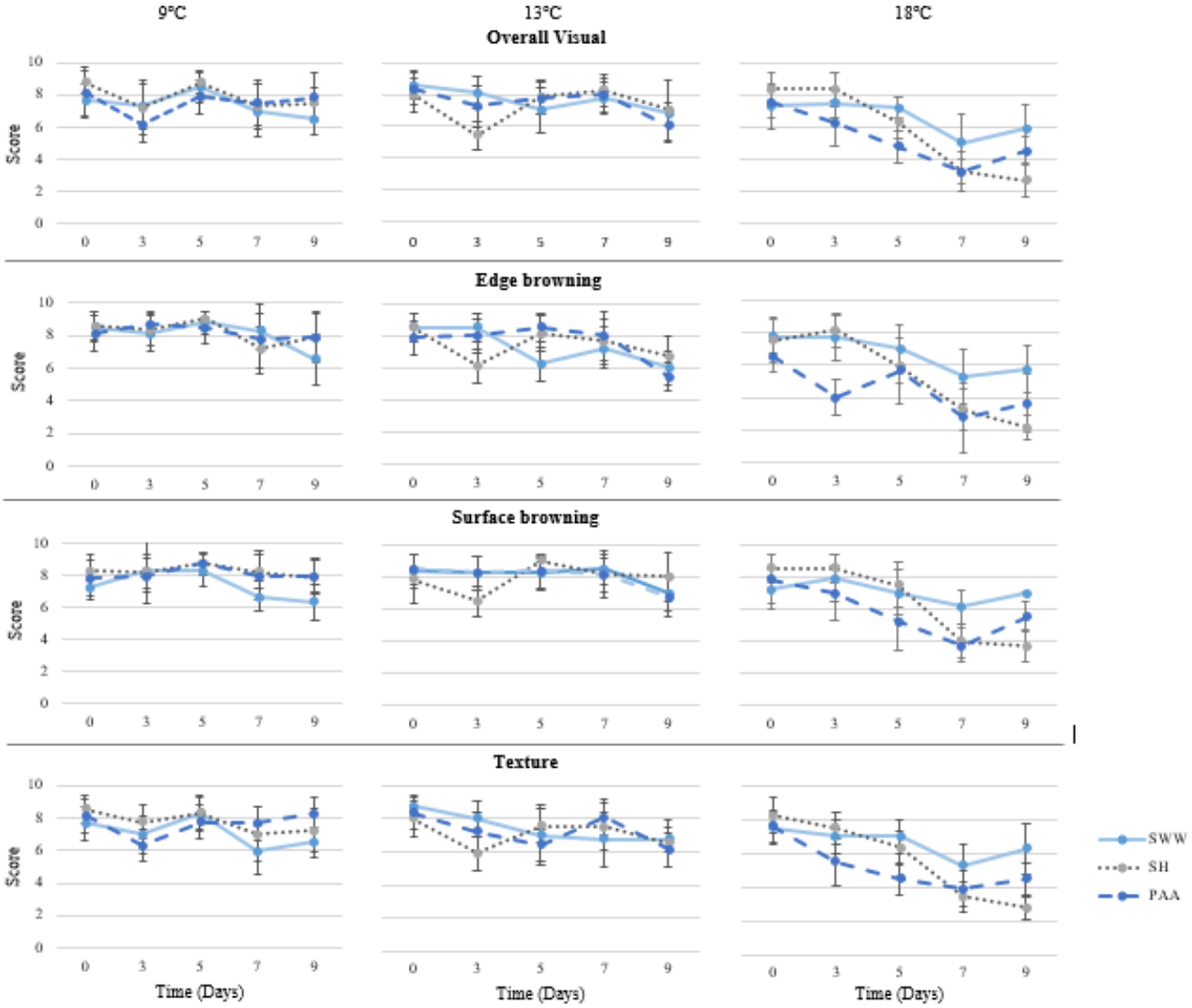


Figure 5.4. Sensory quality evaluation of fresh-cut lettuce washed with Simulated Wash Water (SWW) and disinfectant solutions, stored under modified atmosphere conditions at 9, 13, and 18 ºC for 9 days.

Browning on samples stored at 18 ºC was lower on the edges and surface of lettuce pieces treated with PAA compared to those disinfected with SH (Figure 3). This result may be due, as indicated by Vandekinderen et al., (2009), to an increased respiration rate induced by SH in fresh-cut iceberg lettuce, whereas the PAA effects would be less relevant. It is important to note that browning in lettuce pieces can be caused by several factors. Environmental stress, temperature, and vegetable tissue damage, as well as the use of oxidizing agents for disinfection can trigger an increase in the activity of phenylalanine ammonia lyase (PAL). Upon PAL activity induction, phenolic compounds continue to accumulate. Such molecules are natural substrates for oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase (POD), which give rise to quinones that polymerize producing brown pigments and discoloration at the cut edges of lettuce (García et al., 2019; Hunter et al., 2017; Liu et al., 2021; Taranto et al., 2017). Furthermore, as mentioned by García et al. (2019), it is accepted that browning development could be affected by both the constitutive phenolic compounds (limited or absent in lettuce midrib tissues) that are substrates of PPO, and those synthesized by PAL as a response to the wound signal. This could explain the lower browning found, in this work, on the lettuce surface, with respect to that on the edges.

The treatments evaluated in this work did not produce any anomalous or unpleasant odor (data not shown), which was in line with the observations reported by Lopez-Galvez et al. (2013) and Zhang & Yang (2017).

In general, the panelists' assessment apparently shows a direct correlation between OVQ and lettuce browning and dehydration, showing better results in the samples treated with PAA and SWW than in those treated with SH. In this regard, it is important to highlight that at the time of purchase, consumers base their choice mainly on the OVQ, a combination of quality features that can be judged through the package (James et al., 2010).

5.4.4 *Salmonella* growth potential in fresh-cut lettuce washed with water and disinfectant solutions

The growth capacity of *Salmonella* in lettuce after washing only with SWW, SH and PAA for 60 seconds was evaluated at three different temperatures 9, 13, and 18 °C, for 9 days under modified atmosphere conditions. The initial *Salmonella* concentration at the beginning of storage in fresh-cut lettuce was 3.55 ± 0.34 log CFU/g, 3.24 ± 0.14 log CFU/g, and 3.26 ± 0.14 log CFU/g for the samples treated with SWW, chlorine, and PAA, respectively. At

the end of the storage period (9 days), *Salmonella* concentrations increased at all storage temperatures, as shown in Table 5.3.

Table 5.3. Initial and maximum concentration observed in fresh-cut lettuce washed with Simulated Wash Water (SWW) and disinfectant solutions and stored under modified atmosphere conditions at 9, 13, and 18 ºC for 9 days.

Treatment[1]	T (ºC)	N_0 (log CFU/g)	N_{max} (log CFU/g)
SWW	9	3.55 ± 0.34	5.44 ± 0.56 b
	13		6.31 ± 0.06 ab
	18		6.84 ± 0.41 a
SH	9	3.24 ± 0.14	5.35 ± 0.71 b
	13		6.60 ± 0.37 a
	18		6.67 ± 0.23 a
PAA	9	3.26 ± 0.14	4.63 ± 0.37 c
	13		5.87 ± 0.32 b
	18		7.15 ± 0.15 a

Different letters in the same column indicate significant differences (p<0.05) according to Tukey.
N0: Initial population observed.
Nmax: maximum population observed.
T: temperature of storage.
1 Treatments: SWW: Simulated Wash Water, SH: 25 mg/L chlorine, PAA: 80 mg/L peroxyacetic acid.

In all cases, *Salmonella* populations in the samples increased on the first day of storage and during the subsequent storage days (Figure 5.5). Besides, none of the samples incubated at 9 ºC was able to reach the maximum population density (MPD) during the 9-day storage period (Table 5.3 and Figure 5.5). The values corresponded to 5.44 ± 0.56, 5.35 ± 0.71, and 4.63 ± 0.37 log CFU/g, which were statistically similar among treatments (p=0.2424). Conversely, all samples stored at 13 ºC and 18 ºC reached the MPD for all treatments expressed by the final asymptotic concentration value. At 13 ºC, the final values differed among treatments, corresponding to 6.60 ± 0.37 and 5.87 ± 0.32 log CFU/g for SH and PAA, respectively (p<0.05). Therefore, samples subjected to PAA treatment reached lower concentrations than those observed for SH. At 18 ºC, the maximum levels were statistically identical for both disinfectants (p<0.05).

The growth potential (δ), calculated as the difference between Nf and N0, indicates that, as expected, the highest δ was presented in samples stored at 18 °C. Samples treated with SWW, SH, and PAA showed δ values of 3.14 ± 0.54, 3.43 ± 0.35, and 3.89 ± 0.29 log CFU/g, respectively, which were statistically similar (p=0.1372).

5.4.5 Influence of washing with water and different disinfectants on the estimated kinetic parameters of *Salmonella* in fresh-cut lettuce

Figure 5.5 shows the fitting curves of the Baranyi and Roberts (1994) growth primary model derived from the *Salmonella* counts observed during 9 days of storage at the three different temperatures. The goodness-of-fit of the obtained models corresponded to R^2= 0.80-0.96 and SE =0.26- 0.50.

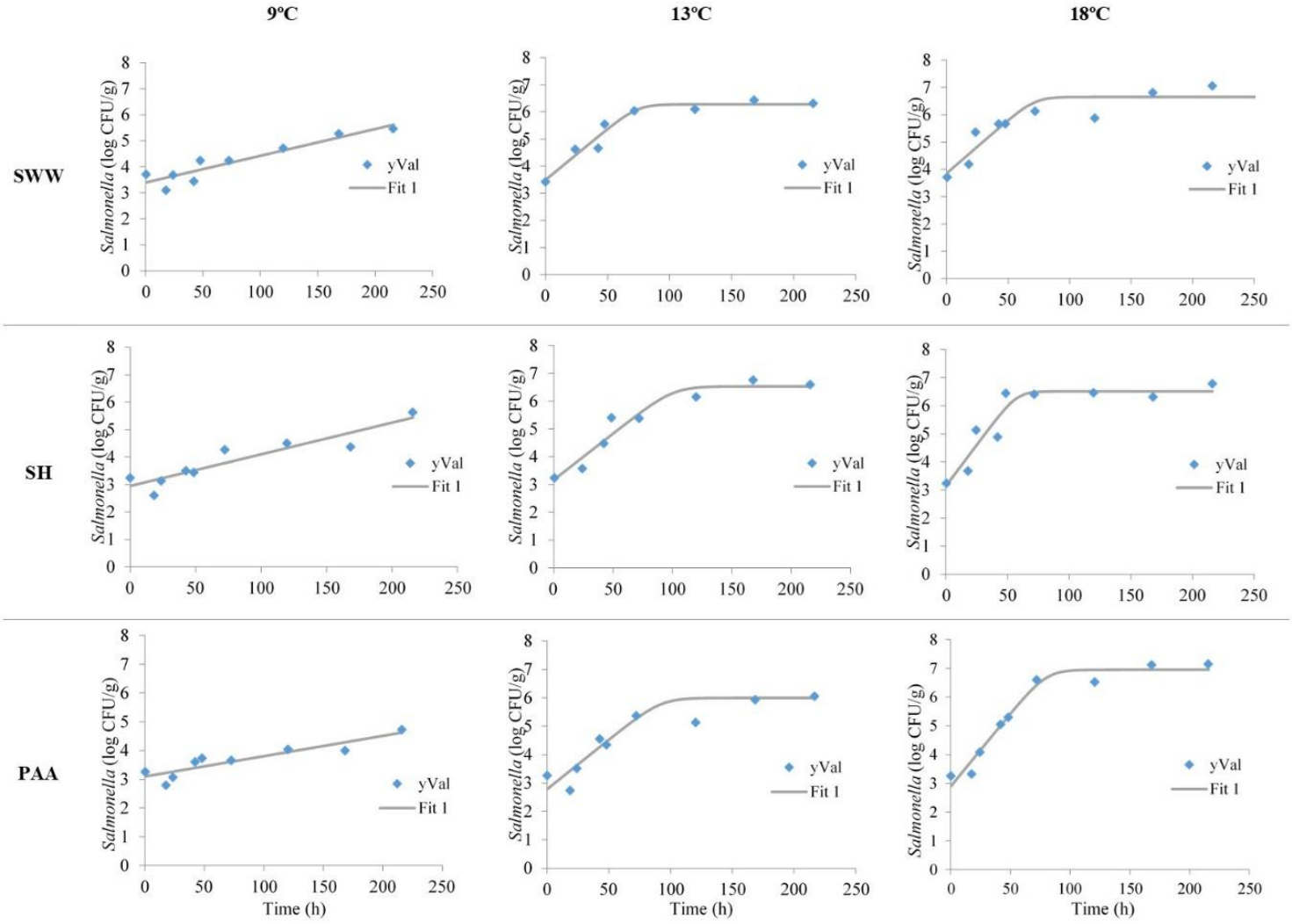


Figure 5.5. Growth curves (points) and fitted growth primary model of Baranyi and Roberts (1994) (solid line) for the *Salmonella* counts observed in fresh-cut lettuce washed with simulated wash water and disinfectant solutions stored under modified atmosphere conditions for 9 days at 9, 13, and 18 ºC.

Table 5.4 presents growth rate obtained from both the primary models and the parameters estimated from the selected secondary models. It is noteworthy that the model showed no lag time (λ) for all treatments (Figure 5.5), including those using disinfectants, which is in agreement with other studies reporting that lag time was very short or absent. For instance, Ndraha et al., (2022) reported no lag time for all *Salmonella* strains at temperatures ranging from 10 ºC to 25 ºC. Similarly, in the study by Tarlak et al. (2020), *Salmonella* Reading showed no lag time in fresh-cut lettuce packaged in modified atmosphere and stored at 15 ºC. In studies where lag time was observed, it was short and limited to around 24 h (Koseki & Isobe, 2005; Puerta-Gomez et al., 2013; Sant'Ana et al., 2012; Yoon et al., 2014; Young Park et al., 2019). For example, in the study by Park et al., (2019), lettuce inoculated with S. Typhimurium and washed with chlorine (100 ppm) resulted in lag time values ranging from 25.86 to 30.46 h at 10 ºC, 17.20 to 21.56 h at 15 ºC, 8.13 to 12.57 h at 20 ºC, and 2.45 to 6.70 h at 25 ºC. In our study, since the first microbiological analysis was performed at 18 and 24 h, we cannot dismiss the possibility that *Salmonella* had a short lag time that was not reflected in the fitted model.

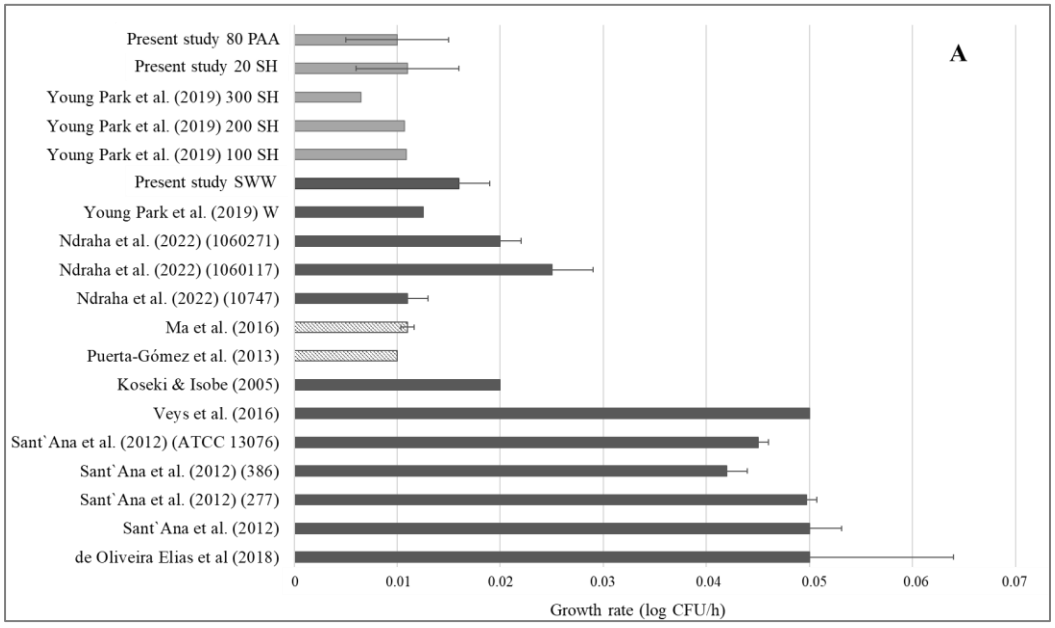
Table 5.4. Growth primary and secondary parameters of *Salmonella* in fresh-cut lettuce washed with Simulated Wash Water (SWW) and disinfectant solutions stored under modified atmosphere conditions at different temperatures (9, 13, and 18ºC).

Treatments[1]	**Primary growth parameters** μ_{max} **(log CFU/h)**[2]			**Secondary growth parameters**[3]	
	9 ºC	**13 ºC**	**18 ºC**	***b***	T_0
SWW	0.016±0.003 a	0.035±0.002 a	0.039±0.009 a	0.00237 ± 0.00065 b	0.67 ± 3.58 b
SH	0.011±0.003 ab	0.034±0.013 a	0.054±0.009 a	0.00465 ± 0.00073 a	6.2 ± 1.25 a
PAA	0.010±0.002 b	0.042±0.015 a	0.048±0.003 a	0.00414 ± 0.00103 a	5.2 ± 2.21 ab

Different letters in the same column indicate significant differences (p<0.05) according to Duncan's test
μmax: maximum growth rate (log CFU/g/h).
1 Treatments: SWW: Simulated Wash Water, SH: 25 mg/L chlorine, PAA: 80 mg/L peroxyacetic acid.
2 estimated parameters ± Standard error.
3 b and T0 are regression parameters as calculated by Bělehrádek-type models represented by Equation 1. (Bělehrádek,1926)

As can be seen from the values in Table 5.4, maximum growth rate increased with temperature. This fact about *Salmonella* growth in fresh-cut vegetables and fruit has been extensively reported in scientific literature (de Oliveira Elias et al., 2018; Ma et al., 2016; Ndraha et al., 2022; A. Singh et al., 2021; Tarlak et al., 2020; Young Park et al., 2019).

Statistical analysis of µmax only showed significant differences between treatments at 9 ºC, where its value was significantly lower in lettuce samples treated with SH and PAA compared to that obtained only for SWW (p<0.05). The µmax values decreased from 0.016 log CFU/h in water to 0.010 log CFU/h in PAA. In the work by Park et al. (2019), *Salmonella* growth in lettuce was also assessed with respect to two disinfectant treatments, reporting a decreased growth when a combination of high concentrations of chlorine and ultrasound was applied to samples compared to control treated with sterile distilled water. To the best of our knowledge, no other work has specifically analyzed the effect of disinfectants on *Salmonella* growth in lettuce or other leafy green produce.



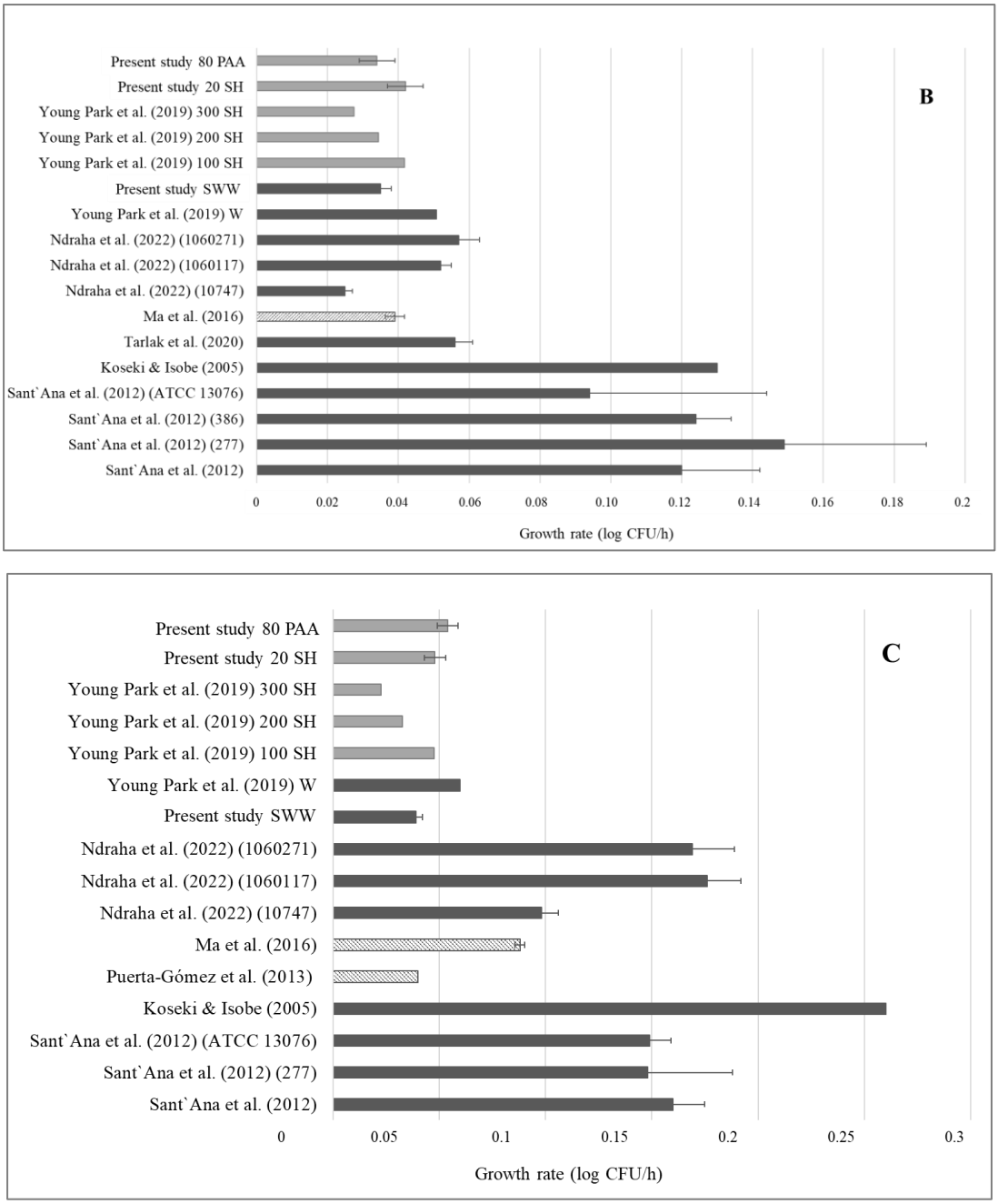


Figure 5.6. Bar plots representing maximum growth rates (log CFU/h) of *Salmonella* strains in lettuce (solid color) and other produces (stripped color) reported by different studies and our study at 9-10 ºC (A), 13-15 ºC (B), and 18-20 ºC (C). Bars with clear grey color stands for those without any disinfectant treatment after the pathogen inoculation.

The growth rate values obtained in our study were compared with literature data from lettuce and similar produces (Figure 5.6). The graphical representation was made considering different temperature ranges to facilitate the comparison of data from similar or very close

temperature values. At first sight, the kinetics of *Salmonella* exposed to disinfectants (this study and that of Park et al., 2019) tended to be lower than those in which no sanitizing treatment was applied after *Salmonella* inoculation. These differences were especially evident for the 9-10 ºC range (Figure 5.6A) (de Oliveira Elias et al., 2018; Koseki & Isobe, 2005; Ndraha et al., 2022; Sant'Ana et al., 2012; Veys et al., 2016; Young Park et al., 2019). However, this trend should be interpreted with caution, as it is limited to the presence of only two studies that include the effect of disinfectant on *Salmonella* growth, since most of the studies do not include a specific design to test this factor. Therefore, the growth rates from studies that did not consider disinfectant were the majority. It is particularly interesting that there was one group that showed much higher values than others. The studies by de Oliveira Elias et al. (2018), Sant'Ana et al. (2012) and Veys et al. (2016) reported values around 0.05 log CFU/h for 9-10 ºC, which doubled those values registered in our study and other works (Koseki & Isobe, 2005; Ma et al., 2016; Ndraha et al., 2022; Puerta-Gomez et al., 2013; Park et al., 2019). Due to the differences between studies, it is difficult to provide any explanation or cause. Studies may differ in temperature, strains, background microbiota, packaging conditions, and inoculum size, among other experimental factors. In addition, several studies have shown that growth kinetics could differ among pathogenic strains (Coleman et al., 2003; Lianou & Koutsoumanis, 2011; Sant'Ana et al., 2012).

The Bělehrádek-type models were used to describe growth rate dependence on temperature. However, the best performance according to SE was obtained for m=1 and without transformation of μmax ($R^2 > 0.83$, SE <0.03). The notional temperature (T0) usually differs from the actual minimum temperature (Ross & Dalgaard, 2004), although it can be considered an intrinsic property of the bacterial population (Ratkowsky et al., 1983). These values were in the range of the values reported in similar works on *Salmonella* and lettuce (Table 5.5) (Koseki & Isobe, 2005; Ndraha et al., 2022; Sant'Ana et al., 2012; Veys et al., 2016), and remain in line with the reported actual minimal growth temperature for these pathogens, which is around 4- 5 ºC (FDA, 2001), even though there are also studies reporting lower and negative values for T0 (de Oliveira Elias et al., 2018; Xiao et al., 2021).

Table 5.5. Parameters of the root-square model (b and T0) for growth rate of *Salmonella* in lettuce sample at different storage temperatures.

Treatment[1]	Temperature range (◦C)	*b*	T_0	Reference
SWW	9 to 18	0.0024 ± 0.0006	0.67 ± 3.59	Present study
SH	9 to 18	0.0047 ± 0.0007	6.25 ± 1.26	Present study
PAA	9 to 18	0.0041 ± 0.0010	5.24 ± 2.21	Present study
Untreated	10 to 25	0.028	8.805	Ndraha et al. (2022)
Untreated	10 to 25	0.028	5.466	Ndraha et al. (2022)
Untreated	10 to 25	0.027	5.477	Ndraha et al. (2022)
Untreated	10 to 25	0.033	4.96	Koseki & Isobe (2005)
Washed/tap water	7 to 30	0.0178	6.65	Sant´Ana et al. (2012)
Untreated	5 to 37	0.027	5.42	Veys et al (2016)
Untreated	5 to 25	0.0339	1.92	de Oliveira et al. (2018)
Untreated	7 to 30	0.018	-7.6	Xiao et al. (2021)
Untreated	7 to30	0.016	-13.5	Xiao et al. (2021)

[1] Treatments: SWW: Simulated Wash Water, SH: 25 mg/L chlorine, PAA: 80 mg/L peroxyacetic acid.

Specifically, in our work, T_0 values were quite similar in SH and PAA treatments, but different from T_0 values obtained from samples treated only with SWW (Table 5.5). Despite these apparent differences, the statistical analysis reported that they were not significantly different (p>0.05). Probably, the high prediction error, derived from the high variability inherent in a complex system such as lettuce, could hinder the analysis from reliably capturing potential differences. Despite the statistical outcome, these results should not be overlooked. From a more conceptual perspective, different T_0 might reflect a different impact of the treatment on the pathogen kinetics, which could also be deduced from the statistical differences between treatments found for μ_{max} at 9 ºC. A lower T_0 would indicate that microorganisms have a higher growth capacity at lower temperatures. The statistical differences (p< 0.05) found for the regression parameter *b* would be in line with this fact, suggesting a different kinetic profile of *Salmonella* in those samples treated with disinfectant.

Figure 5.7 illustrates the fitted secondary models with the estimated regression parameters for each treatment. The observation of the entire temperature range may better reflect the impact of the washing treatment on *Salmonella* growth patterns. The model lines for *Salmonella* exposed to both disinfectants showed similar trends, overlapping over the temperature range tested and with a very similar slope. On the contrary, the model line from the kinetics of *Salmonella* exposed only to SWW presented a completely different pattern, showing a smaller intersection and slope and crossing the SH and PAA lines at around 12 ºC (Figure 5.7). The predicted kinetic behavior would suggest that, at temperatures below 12 ºC, *Salmonella* in fresh-cut lettuce treated with SWW exhibit a better growth capacity (i.e., μ_{max}) than *Salmonella* exposed to SH and PAA. Whereas at temperatures above 12 ºC, the effect would be the opposite, with better *Salmonella* growth capacity in those samples treated with disinfectant (i.e., SH and PAA).

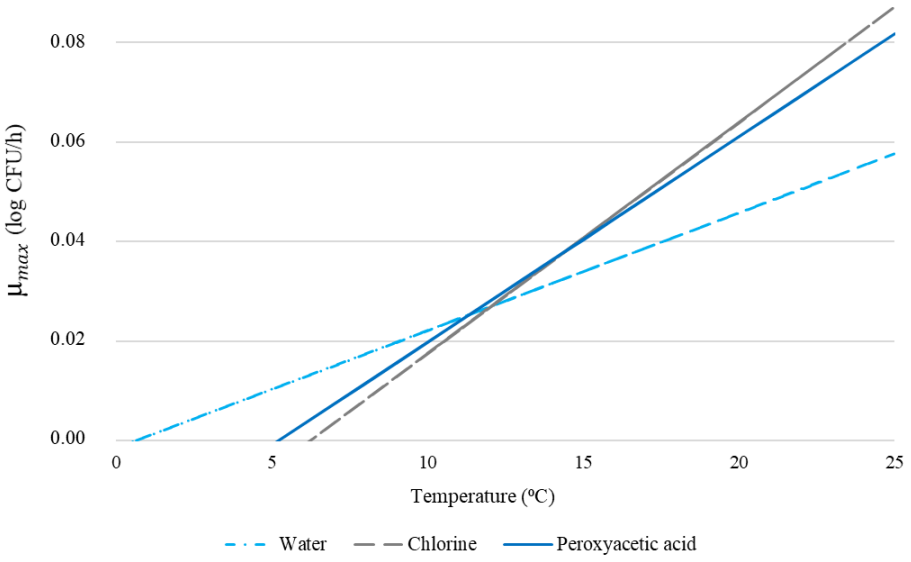


Figure 5.7. Fitted secondary models $[\mu_{max} = b \cdot (T - T_0)]$ describing growth rate dependence on temperature of *Salmonella* in fresh-cut lettuce subjected to disinfection and washing treatments and stored at different temperatures (9-18 ºC) under modified atmosphere.

These results demonstrate that pre-growth stress, in terms of exposure to disinfectants, can determine *Salmonella* kinetics during storage. It is possible that, at lower temperatures, the presence of injured or stressed bacterial cells in lettuce samples treated with disinfectants (SH and PAA) could lead to a slower growth than uninjured *Salmonella* populations in lettuce washed only with water. In this regard, incubation temperature has been shown to have a

significant impact on lesion repair (Liao & Fett, 2005). However, at higher temperatures, the better growth capacity of *Salmonella* cells exposed to disinfectants cannot be explained by the same hypothesis. Some studies have reported that cells can be induced to a filamentous stage by chemical or environmental stress (pH, water activity, desiccation, etc.) (Giotis et al., 2007; Kieboom et al., 2006; Muhandiramlage et al., 2020). When these elongated cells are introduced under more favorable conditions, the filaments could divide and form numerous single cells, rapidly increasing the number of cells and, consequently, the population growth rate (Finn et al., 2013). Nonetheless, this is a hypothesis that needs to be specifically tested considering, in addition, the role of the endogenous microbiota, that, when reduced by disinfectants, could also facilitate pathogen growth. Investigations at molecular and microscopic level are also needed to gain a better understanding of the survival and growth mechanisms of *Salmonella* cells exposed to disinfectants during the washing step of fresh produce.

5.5 Conclusions

In this study, it was observed that the size of the *Salmonella* population in fresh-cut lettuce affected the removal capacity at the washing step, increasing when the population levels in the product were higher (≥ 5.5 log CFU/g). In addition, it was proven that PAA can be an effective alternative for the disinfection of *Salmonella* in fresh-cut lettuce, producing similar reduction levels to chlorine but with slightly better performance in reducing the produce deterioration during storage.

The modeling approach based on the Belehderek-type models could satisfactorily describe *Salmonella* kinetics over the temperature range studied (9-18 ºC). Interestingly, the close examination of the models and their parameters (T0 and b) unveiled a remarkable impact of chlorine and peroxyacetic acid on *Salmonella* kinetics during storage, reducing the growth rate at lower temperatures (≤ 12 ºC) but increasing it at higher temperatures compared to fresh-cut lettuce treated only with water. Further research will be needed to better understand the physiological and biological mechanisms caused by the disinfectant responsible for the observed kinetic behavior.

The data and models developed in this study will be crucial to describe these interactions in a risk assessment framework applied to fresh-cut produce, providing more complete and accurate risk estimates.

Acknowledgements

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5.6 References

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CAPÍTULO 6

6 Capítulo 6: Evaluación de las prácticas aplicadas en la elaboración de vegetales listos para el consumo en Argentina utilizando modelos de microbiología predictiva

Este trabajo se envió a la revista Foods en enero 2023.

Además, parte de los resultados, se compartirán con las industrias participantes.

6.1 Resumen

Este estudio se recopiló información sobre las condiciones y prácticas de procesamiento empleadas en siete industrias de vegetales listos para consumo en Argentina. Un primer análisis de conglomerados indicó la presencia de tres grupos diferentes según las etapas del proceso de elaboración aplicadas. Además, al evaluar los parámetros, métodos y controles utilizados en cada etapa, se identificaron seis grupos diferentes (de siete industrias), lo que indica una gran variabilidad en las prácticas de manejo y los parámetros aplicados en cada paso de procesamiento. El agente desinfectante utilizado en todos los casos fue hipoclorito de sodio, aunque las concentraciones y tiempos de contacto difirieron entre industrias. Cabe destacar que el pH del agua de lavado no fue monitoreado ni controlado en la mayoría de las empresas evaluadas, mientras que sólo una de ellas reportó utilizar ácido cítrico como regulador de pH. Los datos recopilados en este estudio (temperaturas, tiempos, concentraciones) se combinaron con modelos de microbiología predictiva para estimar las concentraciones de *Salmonella* después de la desinfección, durante el almacenamiento y la distribución, clasificando las diferentes prácticas según los niveles finales estimados del patógeno. Este trabajo profundiza en el conocimiento de las operaciones, parámetros y condiciones de procesamiento de vegetales listos para consumo en Argentina, pudiéndose utilizar en estudios de evaluación del riesgo microbiológico.

Evaluation of ready-to-eat vegetables processing practices in Argentina industries using predictive microbiology models

Abstract

This study gathered information on the processing conditions and practices employed in seven companies producing ready-to-eat (RTE) vegetables in Argentina. A cluster analysis

indicated the presence of three different groups according to the stages of the preparation process of RTE vegetables. In addition, by evaluating the parameters, methods, and controls used in each stage, six different clusters (out of 7 processing plants) were identified, indicating a great variability in the handling practices and parameters used in each processing step. The disinfectant agent applied in all cases was sodium hypochlorite, though concentrations and application times differed among companies. It is noteworthy that the pH of wash water was not monitored or controlled in most of the companies evaluated, while only one company reported using citric acid as a pH regulator. The data collected in this study (i.e., temperatures, times, concentrations) were combined with predictive microbiology models to estimate *Salmonella* concentrations after chlorine washing, during storage and distribution, and to rank the different practices according to the final estimated *Salmonella* levels. The results of this study provide a deep knowledge of the RTE vegetable processing operations, parameters, and conditions in Argentina, which can be applied to microbiological risk assessments.

6.2 Introduction

The World Health Organization (WHO) recommends the consumption of at least 400 g of fruits and vegetables per day for a healthy diet (WHO, 2018). In addition, the Dietary Guidelines for the Argentine Population recommend the daily consumption of five servings of fruits and vegetables in a variety of types and colors. However, it has been reported that only 6% of the Argentine population follows this recommendation (Instituto Nacional de Estadística y Censos - INDEC & Secretaría de Gobierno de Salud de la Nación, 2019).

Many factors contribute to consumers not incorporating fruits and vegetables into their diets, including socioeconomic status, availability of and accessibility to quality products, educational level, and, in some cases, the time required to prepare these foods (Adrogué & Orlicki, 2019; Giacobone et al., 2018). The horticultural sector in Argentina is committed and challenged to optimize its production to promote an increase in vegetable consumption, not only in terms of quantity and diversity but also in terms of quality.

While most vegetables are marketed as commodities or in bulk, another part is used for canning and dehydration. However, the production of fresh and RTE products is still an emerging segment in Argentina, with little development in the agro-industrial sector. These products require industrial processing that involves steps including selection and

classification, size reduction, washing-disinfection, centrifugations, and packaging. They are marketed as products for direct consumption in homes, restaurants, hotels, food chains, and catering services, among others (Rojas-Graü et al., 2011). In countries such as Spain, the production of fresh and RTE foods has been widely consolidated, offering the consumer healthy, fresh, and easy-to-consume or prepare foods, with high organoleptic quality (Galizio & Diaz, 2020; Ma et al., 2017; Mir et al., 2018; Yousuf et al., 2020).

It is important to highlight that RTE vegetables are consumed raw, without being subjected to home disinfection steps, which can completely eliminate pathogen contamination. Therefore, these products are most frequently implicated in foodborne outbreaks, which have been primarily related to contamination with pathogens such as *Escherichia coli* O157:H7 or *Salmonella enterica* (Callejón et al., 2015; EFSA, 2017; González et al., 2017; Moretti & Rodríguez, 2020).

The production chain of RTE vegetables is complex and encompasses several critical steps in which food safety may be affected from a microbiological point of view (Pang et al., 2017, Bozkurt, Hayriye, 2021). Hence, a systematic approach including all aspects of the production chain from "farm to table" is required, starting with the quality of the raw material, the effectiveness of washing, disinfection, and cross-contamination control through production, packaging, transport, and storage conditions (Beharielal et al., 2018; Castro-Ibáñez et al., 2016; Goodburn & Wallace, 2013; Koseki, 2014).

The application of predictive microbiology models allows estimating the behavior of pathogenic microorganisms under specific environmental conditions along the food production chain, storage, and distribution (Allende et al., 2022; Pérez-Rodríguez & Valero, 2013; Ross et al., 2014) . Therefore, appropriate predictive models can be used to predict the growth, survival, and inactivation of microorganisms during the production of RTE vegetables to understand the effect of the conditions at each processing step (e.g., temperature, time). In this sense, predictions can be useful for evaluating possible control measures or modifications of process parameters to prevent the exposure of consumers to microbial hazards.

Knowledge of the practices used in the companies processing RTE vegetables can help to understand, prevent, and reduce contamination with pathogens throughout the production chain. Therefore, the aims of this study were: i) to collect information on the practices,

parameters, and methods used in seven RTE vegetable processing companies from different provinces of Argentina; and ii) to use predictive models to estimate *Salmonella* concentrations in RTE vegetables after chlorine washing in the processing plant, during end-product storage in the plant, and during distribution.

6.3 Materials and methods

6.3.1 Participating companies

Ten major RTE vegetables companies (processing plants), located in different regions of Argentina, were contacted for this study. The first contact was made through the e-mails published in their websites and then, through direct communication with the Quality managers of each company. The survey was anonymously.

6.3.2 Survey

A questionnaire regarding the processing steps of RTE leafy vegetables and their parameters was elaborated, considering the most important factors affecting microbial behavior in these types of products (e.g., temperature, time, sanitizer concentration ranges). The final version of the survey consisted of 28 questions with multiple answers, which were subdivided into six sections: 1) Description of the company and the processing steps carried out therein; 2 and 3) Data on the washing step and disinfection of RTE-leafy greens; 4) Data on the packaging conditions; 5) Data on post processing in-plant storage; and 6) Data on transport and distribution of the RTE leafy-green vegetables.

The questionnaire was emailed to the Quality managers of the ten Argentine companies via a GoogleForms® link in March 2021.

6.3.3 Data analysis

The Excel software (Excel® 2016, Microsoft Office) was used for data and statistical analysis as well as for the graphical representation of the data derived from the answers to the questionnaire. A cluster analysis was carried out using the InfoStat software, version 2017 (Grupo InfoStat, Argentina) (Di Rienzo J.A. et al., 2017), to identify the homogeneous groups among the surveyed companies considering the processing steps and conditions adopted by them.

6.3.4 Simulation scenarios using predictive models in the processing and conditioning stages

To evaluate quantitatively the impact of the processing steps, conditions, and practices adopted by the participating companies on the behavior of *Salmonella* in RTE leafy green vegetables, predictive models were used. Two models were selected for the following applications.

First, to evaluate the disinfection efficacy during the washing of fresh-cut lettuce in the presence of *Salmonella*. The model used to this end corresponds to the polynomial model by Cuggino et al., (2020), developed on the basis of the Response Surface Methodology, which describes the *Salmonella* inactivation during chlorine washing as a function of four independent variables: free chlorine concentration (FCC, 0-200 ppm), contact time (30-110 s), cutting size (9-21 cm^2), and benzyl isothiocyanate concentration (BITC, 0-80 ppm). It is important to highlight that the data for this model was obtained using the model water at 4 ºC and water:lettuce ratio of 8.5 L/kg, therefore, these parameters were considered when evaluating the scenarios.

Second, to evaluate the growth potential of *Salmonella* in the post-processing steps of fresh-cut lettuce. To do so, the secondary model by Cuggino et al. (2022, under review) was coupled with the primary model by Baranyi and Roberts (1994). Briefly, to develop this secondary model, growth data over time were obtained in artificially contaminated fresh-cut lettuce at different storage temperatures (9-18 °C). The *Salmonella* growth parameters (i.e., growth rates, maximum population density) were estimated by fitting the Baranyi and Roberts (1994) model to the growth data. Then, the secondary model by Ratkowsky et al., (1982) was used to relate *Salmonella* growth rates to the environmental temperature.

The abovementioned predictive models were implemented in the user-friendly MICROHIBRO software (www.microhibro.com) to be used for simulations. The initial level of *Salmonella* contamination in the lettuce was set to be 3 log CFU/g, that is the highest level that can be found in this type of product (Cuggino et al., 2020; Sant'ana et al., 2011). The parameters used as inputs for the predictive models to evaluate the different scenarios represented by each company were: the average concentration of the disinfectant (ppm) applied during the washing operation, the average disinfection time (s), and the cut size of the pieces of leafy vegetables (cm^2), which varied among companies according to their answers to the questionnaire. The BITC was set to zero, since none of the companies apply

it in the disinfection process. On the other hand, for the storage temperature (ºC) and distribution parameter, the highest temperature in the range of temperatures provided by the companies was selected. Therefore, the simulations using the predictive models considered the worst-case scenarios in the disinfection and storage stages.

6.4 Results and Discussion

Seven of the ten companies contacted answered the survey. They were identified with the letters A to G. Three of them are located in the province of Córdoba; two are located in Buenos Aires; one, in Mendoza; and one, in Santa Fe.

6.4.1 Description of the preparation stages of ready-to-eat leafy vegetables

In all the companies evaluated, the processing stage for the production of RTE leafy vegetables included common processing steps, such as the selection and classification of the raw material, cutting process, washing-disinfection and centrifugation/drying. Moreover, the conditioning stage included packaging, storage, and transport operations. A general process flow diagram, representative of the RTE leafy vegetable production in Argentina, was developed based on the information provided by the seven participating companies (Figure 6.1). The preceding stages are necessary for the transformation of vegetables into ready-to-eat foods (Jideani et al., 2017).

Despite the similarities in the processing and conditioning stages, it should be noted that, in some companies (C, D, E, and F), the stages of the process are totally automated while in others (A, B and G) some stages are manual.

Figure 6.1.General process flow diagram of the elaboration of RTE leafy vegetables in Argentina.

6.4.2 Assessment of the elaboration and conditioning process of RTE leafy vegetables in Argentine companies

In order to identify similarities or differences between the seven processing plants studied, a cluster analysis was carried out applying the information obtained from the survey in relation to the processing stages of RTE leafy vegetables. The results of the cluster analysis the practices observed in the seven plants showed three major homogeneous groups, namely groups 1-3 (Figure 6.2). Group 1, comprising company B, showed a stark difference compared to the other groups, since it does not perform pre-washing, pre-cooling or rinsing stages during the processing of leafy greens. Group 2, comprising companies D, E, F, and G, reported performing all the same processing stages (shown in Figure 6.1). Finally, Group 3, comprising companies A and C, differ from company B in that they perform a rinsing stage, and from Group 2 in that they do not perform the pre-washing and pre-cooling stages.

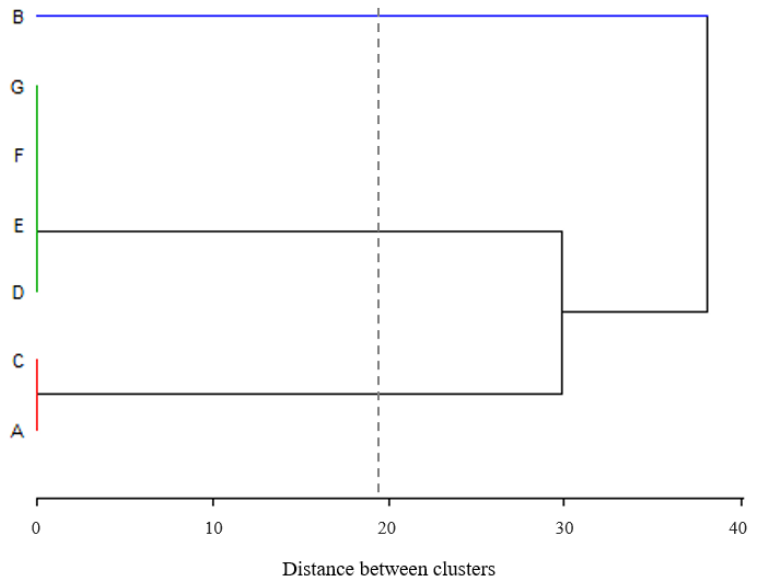

Figure 6.2. Cluster analysis for the stages performed in the processing of ready-to-eat leafy vegetables in seven Argentine companies.

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On the other hand, an analysis of the parameters, methods, and controls used in each stage was carried out, which resulted in six different homogeneous groups, indicating a great variability between the practices and methods used by the different companies (Figure 6.3).

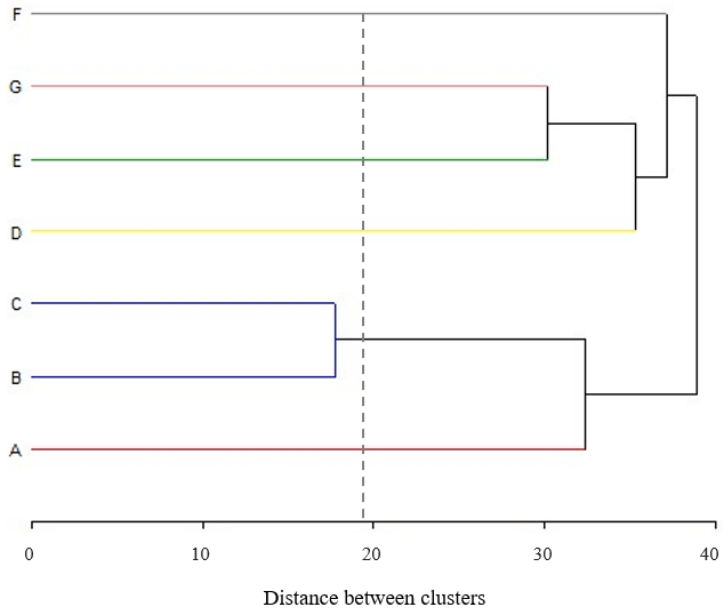

Figure 6.3. Cluster analysis of the parameters and methods used for the processing stages of ready-to-eat leafy vegetables in seven Argentine companies.

The similarities and differences found in the processing and conditioning stages are detailed below.

6.4.3 Processing stages: selection and classification, cutting, disinfection, and centrifugation process

When analyzing in detail the common steps performed by the seven companies, differences were observed in some specific parameters and in the processing operations. For example, all seven companies perform the cutting operation. However, in companies A, B, C, D, E, and F, lettuce is cut prior to disinfection, while in company G, lettuce is first disinfected and then cut. In relation to the cut size of the vegetables, it varies from 9 to 18 cm^2, as shown in Table 1.

Another common step among all surveyed companies is disinfection, but the parameters used vary between companies. For example, the results of the survey show that the temperature of the water used for the disinfection process is greater than or equal to 8 ºC, and, in some cases, the wash water at room temperature is used (Figure 6.6). As indicated by Gil et al., (2014), leafy greens should cool quickly (less than 90 minutes) after harvest. This would allow the

decrease of microbial growth in the case of contamination, and can be partially achieved by using wash water at low temperatures (Maffei et al., 2016; Meireles et al., 2016).

Also, all participating companies use sodium hypochlorite as a disinfectant in the wash water. Sodium hypochlorite is the most widely used disinfectant in the fresh produce industry (De Corato, 2019; Gil et al., 2009; Meireles et al., 2016; Van Haute et al., 2013; Weng et al., 2016) due to its relatively low price, simplicity of application, and broad spectrum of antimicrobial activity (Chen & Hung, 2017; Ramos et al., 2013). On the other hand, the sodium hypochlorite concentration used and the disinfection time differ among companies, as shown in Figure 4. No correlation was observed between chlorine concentration and washing time performed by the participating companies (Figure 6.4).

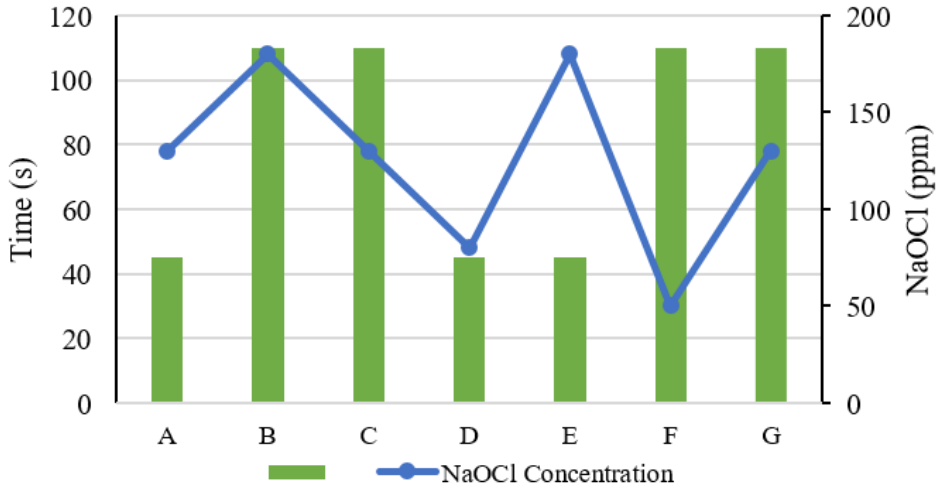

Figure 6.4. Sodium hypochlorite concentration and washing time used in seven ready-to-eat vegetable processing companies in Argentina.

To optimize the disinfection process, knowing the minimum doses of chlorine required to prevent cross-contamination in the wash water while minimizing the formation of by-products (Gómez-López et al., 2014; Zhou et al., 2014) is important. Therefore, to ensure the effectiveness of the disinfection system, continuous monitoring of the disinfectant concentration used becomes necessary (López-Gálvez et al., 2019). In addition, the use of high chlorine concentrations leads to higher concentrations of disinfection by-products (DBPs) in the wash water (López-Gálvez et al., 2019), which should be a controlled parameter.

The results of the survey indicate that, in a few companies, the wash water pH is controlled (Table 6.1). It should be noted that there is much research showing that pH measurement is an essential parameter to control since the effective action of chlorine is highly dependent on pH, and its highest efficiency is at pH 7.5 (Chen & Hung, 2017; Ramos et al., 2013). It should also be highlighted that only company F adds citric acid as a pH regulator.

Table 6.1. Parameters of the processing and conditioning stages of RTE leafy vegetables of seven Argentine companies

Parameter	A	B	C	D	E	F	G
Cut size (cm^2)	16	12	12	9	16	18	16
pH Control/pH	NA	NA	NA	6 to 8	NA	6 to 8	6 to 8
Packaging	Tray with film without vacuum	Tray with film without vacuum	Plastic bags MAP	PET containers with lid	Sealed plastic bags	Sealed plastic bags	Plastic bags MAP
In-plant storage time (h)	12-24	12	12	12	12	12	12
Distribution time (h)	6	6	6	12	6	6	6
Shelf life (h)	144	96	216	144	96	144	192

NA: not applied. MAP: modified atmosphere packaging

Conditioning stage: Packaging, in-plant storage, and transport

Packaging under hygienic conditions, carried out immediately after drying, plays an essential role in the microbiological protection of fresh-cut products (Turatti, 2011; WHO/FAO, 2008). The selection of the material, the conditions generated by the packaging, and the relationship weight/volume of the packaging are very important. In the surveyed companies, differences were observed in the type of packaging material and conditions used. Table 1 shows the packaging type/atmosphere used for RTE leafy vegetables, according to information provided by the companies. Companies A and B use plastic trays with film, without vacuum, while only companies C and G reported the use plastic bags with modified atmosphere packaging (MAP), which is one of the most recommended methods for this type of products (Oliveira et al., 2010; Posada-Izquierdo et al., 2013; Ramos et al., 2013).

MAP has been introduced as an enhancement technology to extend shelf life of RTE vegetables, but it cannot always be implemented in small and medium-sized companies (Arienzo et al., 2020), which choose packaging that requires simple and economic technologies and materials.

Regarding in-plant storage, the finished products are stored for 12 hours in all cases, except for company A, where they are stored from 12 to 24 hours (Table 6.1). After storage, products are normally transported during 6 and up to 12 hours to the point of sale, as indicated by company D. It is important to highlight that companies A and B transport their products during 6 hours at temperatures above 8 ºC.

The shelf life established for RTE vegetables ranges from 96 to 216 hours (4 to 9 days), according to the participating companies (Table 6.1). No correlation was observed between the type and atmosphere of packaging used, in-plant storage and distribution times, and shelf life of the products (Figure 6.5).

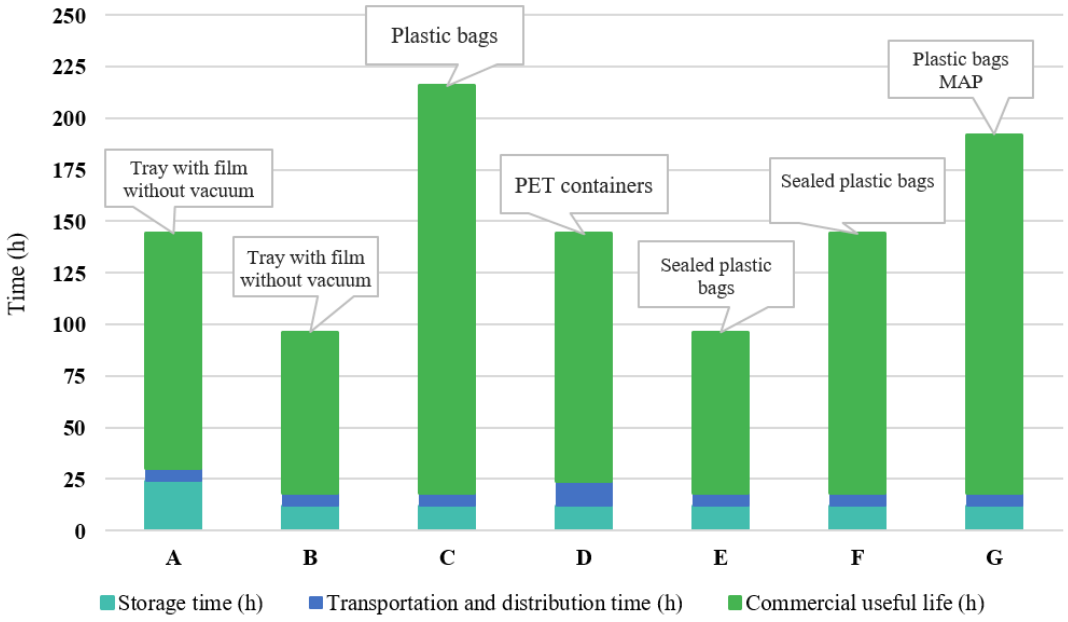

Figure 6.5. Relationship between packaging, storage and distribution time, and shelf life established for RTE leafy vegetables

The shelf life of RTE leafy vegetables can be defined as the time during which the product can maintain an attractive appearance for the consumer in relation to color, hydration, crispness, and absence of browning. Many studies have described the relevance of low temperature as a strategy to avoid/reduce bacterial growth of foodborne pathogens and maintain the organoleptic quality of vegetables (Castro-Ibáñez et al., 2017; de Frias et al., 2018; Luo et al., 2010; Oliveira et al., 2010; Posada-Izquierdo et al., 2013; Sant'Ana et al., 2012; Tsironi et al., 2017).

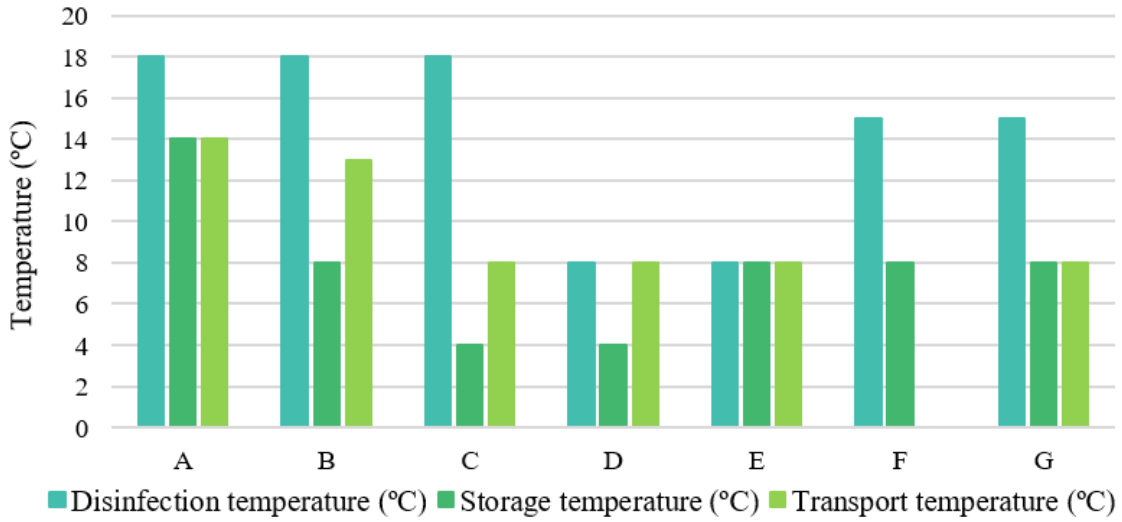

Figure 6.6. Exposure temperatures of leafy vegetables during processing (disinfection) and conditioning (in-plant storage and distribution) in the companies.

The exposure temperatures of leafy vegetables during processing and conditioning are shown in Figure 6.6. In company A, the products are disinfected, stored, and transported at a temperature higher than 8 ºC. On the other hand, companies C, D, E, F, and G store and distribute their products at lower temperatures. Temperature is an important factor in inhibiting the growth of microorganisms and lowering the rate of respiration, which is achieved by keeping refrigerated products at temperatures below 4 ºC (EFSA, 2014; Mir et al., 2018; Sant'Ana et al., 2012). Moreover, many studies have shown that temperature abuse (>12 ºC) favors pathogen growth (Arienzo et al., 2020; Kim et al., 2021; Oliveira et al., 2010; Posada-Izquierdo et al., 2013).

In addition, many studies have demonstrated that refrigeration is the most convenient and effective method to preserve organoleptic properties and extend the shelf life of fresh products (Arienzo et al., 2020; Hoffmann et al., 2021; Maffei et al., 2017; Manzocco et al., 2017). However, to maintain a constant low temperature throughout the processing and distribution of RTE vegetables is a challenge and many small companies cannot afford this cost (Castro-Ibáñez et al., 2017).

6.4.4 Evaluation of *Salmonella* levels in fresh-cut lettuce using predictive models

6.4.4.1 Disinfection efficacy using chlorine washing

Seven different scenarios were considered for model simulations: each representing the processing conditions of each participating company. Table 6.2 shows the results obtained from the simulations of the predictive model by Cuggino et al. (2020) for the evaluation of the efficacy of chlorine washing to inactivate *Salmonella* in fresh-cut lettuce.

Table 6.2. Reductions of *Salmonella* according to disinfection parameters set by the companies.

Company	Chlorine concentration (ppm)	Disinfection time (s)	Cut size (cm^2)	Reductions in *Salmonella* (log CFU/g)
A	130	45	16	2.12
B	180	110	12	2.65
C	130	110	12	2.73
D	80	45	9	1.70
E	180	45	16	1.87
F	50	110	18	2.29
G	130	110	16	2.95

As shown in Table 2, the combination of parameters set by company G generates a greater reduction (i.e., 2.95 log UFC/g). On the contrary, the parameters set by company D show the lowest pathogen reduction (i.e., 1.7 log CFU/g). When comparing companies A and G, it is observed that an increase in the disinfection time results in a greater reduction of *Salmonella*. The provisions of Article 925 of the Food Code (CAA, 2017) in Argentina, and the Commission Regulation No. 2073/2005 (EC, 2005) in Spain lays down that the absence (non-detection) of *Salmonella* must be ensured in 25 g of RTE vegetables. Thus, in a worst-case scenario of *Salmonella* contamination at 3 log CFU/g in fresh-cut lettuce, none of the combinations of the parameters set by the seven companies would ensure the absence of the pathogen in 25 g.

All steps of RTE vegetable processing play an important role in the quality of the final product, but the washing-disinfection step is key to achieve a reduction of microbial loads, while removing dirtiness and cell exudates (D'Acunzo et al., 2012). However, if the chlorine concentrations are not well controlled, cross-contamination events during washing may occur

and play a paramount role in the microbiological safety of the final products (Possas & Pérez-Rodríguez, 2022).

6.4.4.2 Salmonella growth potential during in-plant storage and distribution

The effects of in-plant storage and transport temperatures and times on *Salmonella* growth in fresh-cut lettuce following the chlorine washing step were evaluated applying the model by Cuggino et al. (2022, under review). The temperature and storage and distribution times reported by the seven participating companies were used for model simulations (Table 6.3, Figure 6.6). This model has been developed with *Salmonella* growth data obtained from fresh-cut lettuce previously subjected to chlorine washing. The results of model simulations are shown in Table 6.3.

It is important for the evaluation to clarify that the samples were stored in packaging with a modified atmosphere, since the model was developed with those conditions. In addition, as the model was made for temperatures greater than or equal to 9 ºC; a temperature of 9 °C will be considered for the companies that indicated carrying out storage and distribution at T=8 ºC, as this could be a probable scenario in the summer time.

Table 6.3. Estimation of *Salmonella* growth in fresh-cut lettuce under different conditions of in-plant storage and transport

Company	Concentration of *Salmonella* after disinfection[1] (CFU/g)	Storage temperature (ºC)	Storage time (h)	Concentration of *Salmonella* after Storage (CFU/g)	Transport temperature (ºC)	Transportation and distribution time (h)	Concentration of *Salmonella* after Transport (CFU/g)
A	0.88	14	24	1.74	14	6	1.95
B	0.35	8*	12	0.50	13	6	0.69
C	0.27	4	12	0.27	8*	6	0.35
D	1.30	4	12	1.30	8*	12	1.45
E	1.13	8*	12	1.28	8*	6	1.36
F	0.71	8*	12	0.86	NR	6	-
G	0.05	8*	12	0.20	8*	6	0.28

1) Simulations carried out considering an initial *Salmonella* contamination of 3 CFU/g.
NR: not reported.
*T=9ºC.

Companies C and D indicated that the storage temperature of the products in the plant is 4 ºC, in accordance with the recommended storage temperature for these products (FDA, 2008, 2012; Rediers et al., 2009). Therefore, a growth of *Salmonella* would not be expected during

storage in companies C and D. However, since the disinfection treatment was not sufficient to fully eliminate *Salmonella* from their products, a growth of the pathogen is expected during product distribution (Table 6.3). Considering the storage conditions (14 ºC for 24 h), the highest increase in *Salmonella* levels would occur in products from company A (i.e., 0.86 CFU/g), compared to the other companies evaluated. On the other hand, the storage conditions of companies B, E, and F would also favor the growth of *Salmonella*.

After in-plant storage, RTE vegetables are distributed to retailers. Table 6.3 shows that, in all the scenarios evaluated, there would be an increase in the *Salmonella* concentration during the distribution step. In this sense, company G should ensure no risk of cross-contamination with *Salmonella* during storage, since, after this step, the products are distributed at a higher temperature than recommended.

The application of the predictive microbiology models allowed estimating the behavior of *Salmonella* in all the scenarios considered. It also highlights the importance of effectively combining an efficient disinfection step with the optimal control of storage and transport temperature of RTE vegetables to ensure the final quality and safety of the product.

6.5 Conclusion

The data collected in this study provide a valuable knowledge of the different processes, methods, and practices used along the production chain of ready-to-eat leafy vegetables in Argentina. Furthermore, these data, together with published studies, help to identify and evaluate those factors and process parameters that may affect the innocuousness of fresh processed products. The results of the survey reveal a great variability of practices and parameters used by the different companies producing ready-to-eat leafy vegetables in Argentina, confirming that knowledge and scientific development are often at odds with the industrial reality. The application of mathematical models allowed us to assess the impact of chlorine disinfection, storage, and distribution conditions on the *Salmonella* levels in ready-to-eat lettuce, considering the conditions reported by fresh produce companies. Although chlorine disinfection is a crucial step for controlling pathogens in these products, model simulations confirmed that it is not enough to fully eliminate the pathogen, since it can grow during subsequent the storage and distribution stages. Despite the limited public health data available related to diseases transmitted by ready-to-eat leafy vegetables, this work reflects

the contribution of surveys and predictive microbiology tools to decision-making and the evaluation of control measures in the fresh produce industry.

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CAPÍTULO 7

7 Capítulo 7: Conclusiones

A continuación, se exponen las principales conclusiones de la presente Tesis Doctoral:

Primero. De acuerdo con la literatura revisada, la incorporación en la dieta de vegetales listos para consumo ha aumentado en los últimos años. Este aumento se debe a las características de estos productos: alta calidad organoléptica, alto valor nutricional y facilidad de consumo o preparación. Además, mediante el análisis de informes de producción, comercialización y consumo, se observó que la aceptación de este tipo de producto no es igual en Argentina y en España. Por otro lado, se observó que la industria dedicada al procesamiento de estos alimentos en Argentina, es muy reciente y tiene un marcado potencial de crecimiento (Capítulo 1).

Segundo. Los vegetales listos para consumo se encuentran entre los productos frescos más frecuentemente implicados en brotes de origen alimentario, relacionados principalmente con la contaminación por *Salmonella* entérica, *Escherichia coli* O157:H7, o *Listeria monocytogenes*. Estos patógenos pueden estar presentes en la materia prima, en el agua de riego o en el suelo. Además, pueden llegar al alimento por contaminación directa o indirecta de los manipuladores, las superficies o equipos utilizados durante su transformación. Asimismo, la contaminación puede darse durante el envasado, su distribución y almacenamiento en góndolas. Por lo tanto, se concluye que la seguridad microbiológica de los vegetales listos para consumo requiere de un enfoque sistemático que incluya todos los aspectos de la cadena de producción, desde la "granja hasta la mesa", aplicando un Sistemas de Gestión de Calidad y Seguridad en la producción (Capítulos 1 y 3).

Tercero. La evaluación microbiológica de los puntos críticos de control del proceso de elaboración de rúcula lista para consumo en una industria argentina, indica que el proceso de desinfección aplicado logró disminuir el número de microrganismos mesófilos y eliminar, de manera eficiente, las poblaciones de *E. coli* de la materia prima. La presencia de este microorganismo indicador de higiene resalta la relevancia que tiene el control de los proveedores sobre el agua de riego de la materia prima (Capítulo 3).

Cuarto. La evaluación de alternativas al uso o disminución de cloro como desinfectante demostró que el isotiocianato de bencilo (BITC), en combinación con cloro, podría mejorar ligeramente el rendimiento de la desinfección. Este efecto podría estar relacionado con la reducción del pH del agua que se genera por acción directa del isotiocianato de bencilo. Además, las combinaciones de hipoclorito de sodio y BITC y los parámetros del proceso de elaboración de lechuga lista para consumo (tamaño de corte de las muestras y tiempo de contacto) dieron como resultado una reducción media de *Salmonella* enterica sv Thompson de 2,5 log CFU/g (Capítulo 4).

Quinto. Según el análisis estadístico, las concentraciones de cloro libre y BITC, el tiempo de contacto y el tamaño del corte, ejercieron un efecto significativo en la reducción del patógeno estudiado ($p \leq 0.05$) (Capítulo 4).

Sexto. La máxima capacidad desinfectante del cloro sobre *Salmonella* enterica sv Thompson no se produjo a la máxima concentración estudiada (es decir, 200 mg/L), sino que se observó en el rango de 100 a 150 mg/L de cloro libre. El mismo comportamiento se observó con BITC, cuyo valor óptimo no coincidió con la concentración máxima utilizada (80 mg/L), sino con un valor intermedio, que correspondió a 40 mg/L. El análisis estadístico reveló un efecto lineal positivo del cloro, indicando que a medida que aumenta el nivel de cloro libre, aumenta la reducción del patógeno; y un efecto cuadrático negativo para cloro y BITH, que revela que la reducción máxima no se produjo a la concentración máxima del desinfectante (Capítulo 4).

Séptimo. El análisis estadístico de un diseño compuesto central (DCC) indicó que la combinación óptima de las diferentes variables que produjo la mayor reducción de *Salmonella* enterica sv Thompson (3,2 log UFC/g) en lechuga iceberg, correspondió a un tamaño de corte del producto de 15 cm^2, lavado durante 110 s en agua modelo que contenía 160 mg/L de cloro libre y 40 mg/l de BITC (Capítulo 4).

Octavo. El modelo polinomial desarrollado permitió estimar, de manera satisfactoria, el efecto del hipoclorito de sodio y BITC en combinación con parámetros del proceso de desinfección sobre la reducción de *Salmonella* enterica sv Thompson. Además, el modelo se

pudo aplicar para desarrollar un estudio cuantitativo de evaluación de la exposición. El estudio realizado en esta tesis, indicó que la desinfección es un paso crucial para eliminar el patógeno, aunque en algunos condicioness, no pudo eliminarse *Salmonella* completamente del lote de evaluado, lo que permitiría que el patógeno puediera crecer durante el almacenamiento y distribución (Capítulo 4).

Noveno. Se describió satisfactoriamente el crecimiento de *Salmonella* enterica sv Thompson en lechugas iceberg cortadas y tratadas con diferentes desinfectantes y almacenadas a 9, 13 y 18ºC, durante 9 días, utilizando el modelo de Baranyi y Roberts. Además, se generaron modelos empíricos, basados en el enfoque Belehderek, que describió satisfactoriamente la cinética de *Salmonella* en el rango de temperatura estudiado (Capítulo 5).

Décimo. El estudio de los parámetros del modelo de crecimiento de *Salmonella* enterica sv Thompson (T0 y b) reveló un impacto del cloro y el ácido peroxiacético en la cinética del patógeno durante el almacenamiento, reduciendo la tasa de crecimiento a temperaturas más bajas (≤ 12 ºC) pero incrementándola a temperaturas más altas, en comparación con la lechuga recién cortada tratada solo con un agua modelo (Capítulo 5).

Undécimo. El hipoclorito de sodio es el desinfectante más utilizado en la industria de vegetales listos para consumo, pero existe una tendencia en reducir su concentración y/o sustituirlo. Los resultados de esta tesis han demostrado que el ácido peroxiacético (PAA) puede usarse como una alternativa eficaz para la desinfección de *Salmonella* enterica sv Thompson en lechuga iceberg cortada, produciendo niveles de desinfección similares al cloro, pero con un rendimiento ligeramente mejor en la reducción del deterioro del producto durante el almacenamiento (Capítulo 5).

Duodécimo. En la evaluación de las prácticas y parámetros utilizados en siete industrias procesadoras de vegetales listos para consumo de Argentina, se identificaron seis grupos diferentes, lo que revela una gran variabilidad en el proceso aplicado. El agente desinfectante utilizado en todas las industrias fue el hipoclorito de sodio, aunque la concentración utilizada y el tiempo de aplicación fue diferente, sin observarse una correlación directa. Además, el

pH del agua de lavado no fue monitoreado ni controlado en la mayoría de las empresas, mientras que solo una empresa reportó utilizar ácido cítrico como regulador de pH (Capítulo 6).

Decimotercero. Se realizó una evaluación de la exposición a *Salmonella* spp. en base a los datos recopilados de las industrias argentinas estudiadas. Para ello, el efecto del proceso de desinfección de *Salmonella* en lechugas cortas se estimó utilizando el modelo predictivo polinomial desarrollado en el Capítulo 4, y el potencial de crecimiento del patógeno durante el almacenamiento y distribución se simuló mediante el modelo de crecimiento desarrollado en el Capítulo 5. Los datos y modelos desarrollados en esta tesis serán cruciales para describir interacciones, en un marco de evaluación de riesgos aplicado a productos frescos cortados, proporcionando estimaciones de riesgo más completas y precisas (Capítulo 6).

Decimocuarto. La elaboración de vegetales listos para consumo en Argentina se realiza en grandes industrias, exclusivamente dedicadas a la preparación de vegetales listos para consumo o en pequeñas industrias o establecimientos, que adaptan algún espacio para la transformación de estos productos, como agregado de valor para su producción primaria. Por lo tanto, esperamos que los conocimiento, avances y modelos de microbiología predictiva desarrollados en esta tesis brinden una base cuantitativa que describa el impacto de los procesos, pasos y parámetros utilizados en la elaboración, sobre el riesgo microbiológico en este tipo de alimentos.